



Europäisches Patentamt
European Patent Office
Office européen des brevets

⑪ Publication number:

0 387 701
A1

⑫

EUROPEAN PATENT APPLICATION

⑬ Application number: 90104423.0

⑭ Int. Cl. 5 A61K 37/02, A61K 39/395

⑯ Date of filing: 08.03.90

⑯ Priority: 09.03.89 US 321018
09.03.89 US 321239
09.03.89 US 321237
16.03.89 US 324481
01.09.89 US 401409

⑭ Date of publication of application:
19.09.90 Bulletin 90/38

⑮ Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

⑯ Applicant: Boehringer Ingelheim
Pharmaceuticals Inc.
90 East Ridge P.O. Box 368
Ridgefield Connecticut 06877(US)

⑯ Inventor: Wegner, Craig D.
10 Skyview Drive
New Milford Connecticut 06766(US)
Inventor: Gundel, Robert H.
RR2 Box 74 Hurd's Corner Road
Pawling New York 12564(US)
Inventor: Rothlein, Robert
32 Tammany Trail
Danbury Connecticut 06811(US)

⑯ Representative: Laudien, Dieter, Dr. et al
Boehringer Ingelheim GmbH, Abteilung
Patente
D-6507 Ingelheim am Rhein(DE)

⑯ Use of intercellular adhesion molecules, and their binding ligands in the treatment of asthma.

⑯ The present invention relates to the use of intercellular adhesion molecules (ICAM-1), their functional derivatives, and molecules which bind to them, in the treatment of asthma.

EP 0 387 701 A1

USE OF INTERCELLULAR ADHESION MOLECULES, AND THEIR BINDING LIGANDS IN THE TREATMENT
OF ASTHMA

FIELD OF THE INVENTION

The present invention relates to the use of intercellular adhesion molecules such as ICAM-1 in the treatment of asthma. The invention additionally concerns ligand molecules capable of binding to such intercellular adhesion molecules, and to an assay for detecting agents having therapeutic potential in the treatment of asthma.

DESCRIPTION OF THE RELATED ART

10

A. Cellular Adhesion

Leukocytes must be able to attach to cellular substrates in order to properly defend the host against foreign invaders such as bacteria or viruses. An excellent review of the defense system is provided by Eisen, H.W., (In: Microbiology, 3rd Ed., Harper & Row, Philadelphia, PA (1980), pp. 290-295 and 381-418). Leukocytes must be able to attach to endothelial cells so that they can migrate from circulation to sites of ongoing inflammation. Furthermore, they must attach to antigen-presenting cells so that a normal specific immune response can occur, and finally, they must attach to appropriate target cells so that lysis of virally-infected or tumor cells can occur.

Leukocyte surface molecules involved in mediating such attachments have been identified using hybridoma technology. Briefly, monoclonal antibodies ("MAbs") directed against human T-cells (Davignon, D. et al., Proc. Natl. Acad. Sci. USA 78:4535-4539 (1981)) and mouse spleen cells (Springer, T. et al., Eur. J. Immunol. 9:301-306 (1979)) were identified which bound to leukocyte surfaces and inhibited the attachment functions described above (Springer, T. et al., Fed. Proc. 44:2660-2663 (1985)). The molecules identified by those antibodies were called Mac-1 and Lymphocyte Function-associated Antigen-1 (LFA-1). Mac-1 is found on macrophages, granulocytes and large granular lymphocytes. LFA-1 is found on most lymphocytes (Springer, T.A. et al., Immunol. Rev. 68:111-135 (1982)). These two molecules, plus a third molecule, p150,95 (which has a tissue distribution similar to Mac-1) play a role in cellular adhesion (Keizer, G. et al., Eur. J. Immunol. 15:1142-1147 (1985)). Molecules such as those of LFA-1 family, which are involved in the process of cellular adhesion are referred to as "adhesion molecules."

The above-described leukocyte molecules were found to be structurally similar to one another, and to constitute members of a related family of glycoproteins (Sanchez-Madrid, F. et al., J. Exper. Med. 158:1785-1803 (1983); Keizer, G.D. et al., Eur. J. Immunol. 15:1142-1147 (1985)). This glycoprotein family is composed of heterodimers having one alpha subunit and one beta subunit. Although the alpha subunit of each of the antigens differed from one another, the beta subunit was found to be highly conserved (Sanchez-Madrid, F. et al., J. Exper. Med. 158:1785-1803 (1983)). The beta subunit of the glycoprotein family (referred to as "CD18" family) was found to have a molecular weight of 95 kd whereas the alpha subunits were found to vary from 150 kd to 180 kd (Springer, T., Fed. Proc. 44:2660-2663 (1985)). Although the alpha subunits of the membrane proteins do not share the extensive homology shared by the beta subunits, close analysis of the alpha subunits of the glycoproteins has revealed that there are substantial similarities between them. Reviews of the similarities between the alpha and beta subunits of the LFA-1 related glycoproteins are provided by Sanchez-Madrid, F. et al., (J. Exper. Med. 158:586-602 (1983); Exper. Med. 158:1785-1803 (1983)).

A group of individuals has been identified who are unable to express normal amounts of any member of this adhesion protein family on their leukocyte cell surface (Anderson, D.C., et al., Fed. Proc. 44:2671-2677 (1985); Anderson, D.C., et al., J. Infect. Dis. 152:668-689 (1985)). The condition is known as "Leukocyte Adhesion Deficiency" or "LAD" syndrome. Leukocytes from these patients displayed *in vitro* defects similar to normal counterparts whose CD18 family of molecules had been antagonized by antibodies. Furthermore, these individuals are unable to mount a normal immune response due to an inability of their cells to adhere to cellular substrates (Anderson, D.C., et al., Fed. Proc. 44:2671-2677 (1985); Anderson, D.C., et al., J. Infect. Dis. 152:668-689 (1985)). LAD individuals present clinically with delayed umbilical cord separation, recurring and progressive soft tissue infections, and impaired pus formation, despite a striking blood leukocytosis. Studies of LAD individuals have revealed that immune reactions are mitigated when

phocytes are unable to adhere in a normal fashion due to the lack of functional adhesion molecules of the CD18 family.

Thus, in summary, the ability of leukocytes, especially lymphocytes to maintain the health and viability of an animal requires that they be capable of adhering to other cells (such as endothelial cells). This adherence has been found to require cell-cell contacts which involve specific receptor molecules present on the cell surface of the lymphocytes. These receptors enable a lymphocyte to adhere to other lymphocytes or to endothelial, and other non-vascular cells. The cell surface receptor molecules have been found to be highly related to one another. Humans whose lymphocytes lack these cell surface receptor molecules exhibit defective antibody responses, chronic and recurring infections, as well as other clinical symptoms.

10

B. Asthma: Clinical Characteristics

Asthma is a heterogeneous family of diseases. It is characterized by a hyper-responsiveness of the tracheobronchi to stimuli (McFadden, E.R. et al., In: Harrison's Principles of Internal Medicine, 10th Ed., Petersdorf, R.G. et al., Eds., McGraw-Hill, NY (1983), pages 1512- 1519); Kay, A.B., Allergy and Inflammation, Academic Press, NY (1987); which references are incorporated herein by reference). Clinically, asthma is manifested by the extensive narrowing of the tracheobronchi, by thick tenacious secretions, by paroxysms of dyspnea, cough, and wheezing. Although the relative contribution of each of these conditions is unknown, the net result is an increase in airway resistance, hyperinflation of the lungs and thorax, abnormal distribution of ventilation and pulmonary blood flow. The disease is manifested in episodic periods of acute symptoms interspersed between symptom-free periods. The acute episodes result in hypoxia, and can be fatal. Approximately 3% of the general world population suffers from the disease.

Two types of asthma have been described: allergic asthma and idiosyncratic asthma. Allergic asthma is usually associated with a heritable allergic disease, such as rhinitis, urticaria, eczema, etc. The condition is characterized by positive wheal-and-flare reactions to intradermal injections of airborne antigens (such as pollen, environmental or occupational pollutants, etc.), and increased serum levels of IgE. The development of allergic asthma appears to be causally related to the presence of IgE antibodies in many patients. Asthma patients who do not exhibit the above-described characteristics are considered to have idiosyncratic asthma.

Allergic asthma is believed to be dependent upon an IgE response controlled by T and B lymphocytes and activated by the interaction of airborne antigen with mast cell-bound pre-formed IgE molecules. The antigenic encounter must have occurred at concentrations sufficient to lead to IgE production for a prolonged period of time in order to sensitize an individual. Once sensitized, an asthma patient may exhibit symptoms in response to extremely low levels of antigen.

Asthma symptoms may be exacerbated by the presence and level of the triggering antigen, environmental factors, occupational factors, physical exertion, and emotional stress.

Asthma may be treated with methylxanthines (such as theophylline), beta-adrenergic agonists (such as catecholamines, resorcinols, saligenins, and ephedrine), glucocorticoids (such as hydrocortisone), inhibitors of mast cell degranulation (i.e. chromones such as cromolyn sodium) and anticholinergics (such as atropine).

C. Asthma: Immunological Characteristics

Asthma is believed to involve an influx of eosinophils ("eosinophilia") into the tissues of the lung (Frigas, E. et al., J. Allergy Clin. Immunol. 77:527-537 (1986), which reference is incorporated herein by reference). Eosinophils contain an arginine-rich, strongly basic protein which has been termed "MBP" (Gleich, G.J. et al., J. Exper. Med. 137:1459 (1973)). MBP comprises more than 50% of the granule protein of the eosinophil.

MBP was found to be toxic to normal mammalian cells, and to be expressed at toxic levels in tissues evidencing eosinophilia, such as the lung tissue of asthma patients (Gleich, G.J. et al., J. Immunol. 123:2925 (1975). The linkage between MBP expression and asthma was strengthened by the discovery that the MBP levels found in the sputum of asthma patients was elevated relative to normal individuals (Frigas, E. et al., Mayo Clin. Proc. 56:345 (1981)). Since the discovery of MBP, other cytotoxic eosinophil proteins have been identified (Frigas, E. et al., J. Allergy Clin. Immunol. 77:527-537 (1986)).

Insight into the immunological basis of asthma has been gained from the above-described studies, from bronchoalveolar lavage studies (Godard, P. et al., J. Allergy Clin. Immunol. 70:88 (1982)), and studies of

respiratory smooth muscle denuded of epithelium (Flavahan, N.A. et al., *J. Appl. Physiol.* 58:834 (1985); Barnes, P. J. et al., *Br. J. Pharmacol.* 86:685 (1985)). Although these studies have not led to the elucidation of the mechanism underlying the immunology of asthma, they have led to the development of a generally accepted hypothesis concerning the immunological etiology of the disease (see, Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527-537 (1986)).

The hallmarks of the pathology of asthma are a massive infiltration of the lung parenchyma by eosinophils and the destruction of mucociliary capacity. The "eosinophil hypothesis" suggests that eosinophils are attracted to the bronchus in order to neutralize harmful mediators released by the mast cells of the lung. According to the hypothesis eosinophils are attracted to the bronchi where they degranulate to release MBP and other cytotoxic molecules. Upon degranulation, eosinophils release enzymes such as histaminase, arylsulfatase and phospholipase D which enzymatically neutralize the harmful mediators of the mast cell. These molecules also promote the destruction of the mucociliary apparatus, and thus prevent the clearing of the bronchial secretions, and contribute to the lung damage characteristic of asthma.

Thus, it is believed that asthma is caused by the eosinophils which invade the bronchi in an abnormal response to primary effects of antigen presence. The MBP of the eosinophils damage the epithelial cells of the bronchi. Leukotrienes and Platelet Activating Factor ("PAF") are produced by the eosinophil, and result in bronchial dilation. Molecules (such as MBP) released by the eosinophil can also activate mast cells to release leukotrienes and histamine, thereby causing both bronchospasm and increased eosinophilia.

In view of the clinical importance of asthma, it is highly desirable to identify new or improved therapies for treating asthma patients.

SUMMARY OF THE INVENTION

The present invention relates to the use of intercellular adhesion molecules ("ICAMs"), such as ICAM-1, and the functional derivatives of such ICAMs in the treatment of asthma. The invention further concerns the use of molecules (such as antibodies, or antibody fragments, or receptor molecules) that are able to bind to an intercellular adhesion molecule (or a derivative of an ICAM molecule) in the treatment of asthma. The invention also includes an assay for detecting agents having therapeutic potential in the treatment of asthma.

The invention additionally pertains to a method for treating asthma in a patient which comprises providing to the patient an effective therapeutic amount of an agent selected from the group consisting of:

- (a) an antibody capable of binding to ICAM-1;
- (b) a fragment of the antibody (a), the fragment being capable of binding to ICAM-1;
- (c) ICAM-1, being substantially free of natural contaminants;
- (d) a functional derivative of ICAM-1;
- (e) an antibody capable of binding to a member of the CD18 family of glycoproteins;
- (f) a fragment of the antibody (e), the fragment being capable of binding to a member of the CD18 family of glycoproteins;
- (g) a member of the CD18 family of glycoproteins, being substantially free of natural contaminants;
- and
- (h) a functional derivative of a member of the CD18 family of glycoproteins.

The invention further provides a method of identifying an agent capable of therapeutic potential in the treatment of asthma which comprises administering the agent to a non-human mammal (especially a primate) having received multiple inhalations of an antigen and then measuring any increase in airway responsiveness.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide and amino acid sequence of ICAM-1 cDNA. The first ATG is at position 58. Translated sequences corresponding to ICAM-1 tryptic peptides are underlined. The hydrophobic putative signal peptide and transmembrane sequences have a bold underline. N-linked glycosylation sites are boxed. The polyadenylation signal AATAAA at position 2976 is over-lined. The sequence shown is for the HL-60 cDNA clone. The endothelial cell cDNA was sequenced over most of its length and showed only minor differences.

Figure 2 shows the domain structure of ICAM-1.

Figure 3 shows eosinophil adherence to flat bottom tissue culture plate wells protein-coated with no

stimulus, protein-coated with PAF (10^{-7} M) stimulus, immune complex (IC) coated and stimulus, and non-stimulus, protein-coated with PAF (10^{-7} M) stimulus, immune complex (IC) coated and stimulus, and non-stimulus, protein-coated with no stimulus. Adhered cells were quantitated by a colorimetric assay for eosinophil peroxidase (EPO) as ban EPO units \pm S.D.

Figure 4 shows the effect of various monoclonal antibodies (MAbs) (supernatant 1:4 dilution) on 5 eosinophil adherence to flat bottom tissue culture plate wells coated with immune complexes (IC). Adhered cells were quantitated by a colorimetric assay for eosinophil peroxidase (mean EPO units \pm S.D.). Statistically significant attenuation of adherence is signified by an asterisk.

Figure 5 shows the effect of various monoclonal antibodies (MAbs) (supernatant 1:4 dilution) on PAF (10 $^{-7}$ M) induced eosinophil adherence to LPS (10 ng/ml)-stimulated and glutaraldehyde-fixed endothelium. 10 Adhered cells were quantitated by a colorimetric assay for eosinophil peroxidase (EPO units \pm S.D.). Statistically significant attenuation of adherence is signified by an asterisk.

Figure 6 shows the effects of anti-ICAM-1 (RR1/1), anti-LFA-1 beta (R15.7) and anti-HLA class 1 (W6/32) monoclonal antibodies (supernatant 1:4 dilution) on (A) platelet-activating factor (PAF, 10 $^{-7}$ M) induced eosinophil adhesion to lipopolysaccharide (LPS, 10 ng/ml)-stimulated human umbilical vein endothelium, and (B) eosinophil adhesion to flat bottom tissue culture plate wells coated with Ascaris extract (no stimulus) or immune complex (IC stimulus). Statistically significant attenuation of adherence is signified by an asterisk.

Figure 7 shows the effect of the anti-ICAM-1 monoclonal antibody R6.5 on the (A) airway eosinophil infiltration and (B) increase in airway responsiveness (decrease in methacholine PC₁₀₀) induced by three 20 alternate day inhalations of Ascaris in Ascaris-sensitive cynomolgus monkeys (Macaca fascicularis). Studies with R6.5 treatment are compared to bracketing control studies performed on each animal.

Figure 8 shows changes induced by a single inhalation of antigen in (A) inhaled methacholine PC₁₀₀, (B) bronchoalveolar lavage (BAL) eosinophils, (C) BAL neutrophils, (D) BAL macrophages/monocytes, and 25 (E) BAL lymphocytes. Xsq = Kruskal-Wallis Test (Chi-square approximation). NSD = no significant difference. Each letter represents an individual monkey.

Figure 9 shows changes induced by multiple inhalations of antigen in (A) inhaled methacholine PC₁₀₀ and (B) bronchoalveolar lavage (BAL) eosinophils. Xsq = Kruskal-Wallis Test (Chi-square approximation). Each letter represents an individual monkey.

30 DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. The Immunopathology of Asthma

35 As indicated above, one of the most important and characteristic features of asthma is the extreme (10 to 1000 times normal) sensitivity of the bronchi to inhaled agents (physical, chemical and physiological) (Boushey, H.A. et al., Am. Rev. Respir. Dis. 121:389 (1980); Hargreave, F.E. et al., J. Allergy Clin. Immunol. 68:347 (1981)).

40 The severity of this "airway hyperresponsiveness" (which is measured clinically by determining the responsiveness of the airways to inhaled histamine, methacholine, cold air or to exercise) correlates with the intensity of asthmatic symptoms (Hargreave, F.E. et al., J. Allergy Clin. Immunol. 68:347 (1981); Boulet, L-P et al., J. Allergy Clin. Immunol. 71:399 (1983); Chan-Yeung, M. et al., Am. J. Med. 72:411 (1982)), diurnal variations in peak flow rates (Ryan, G. et al., Thorax 37:423 (1982)) and therapy required (Hargreave, F.E. et al., J. Allergy Clin. Immunol. 68:347 (1981); Juniper, E.F. et al., Thorax 36:575 (1981)).

45 Although airway hyperresponsiveness in asthmatics can remain stable over several years (Juniper, E.F. et al., Thorax 37:288-291 (1982)), responsiveness has been shown to be increased by exposure to allergens (Boulet, L-P. et al., J. Allergy. Clin. Immunol. 71:399-406 (1983), Cartier, A. et al., J. Allergy Clin. Immunol. 70:170-177 (1982), Cockcroft, D.W. et al., Clinical Allergy 7:503-513 (1977), Gundel, R.H. et al., Amer. Rev. Respir. Dis. 131:875-879 (1985), Sotomayor, H. et al., Amer. Rev. Respir. Dis. 130:56-58 (1984)), air pollutants (Golden, J.A. et al., Amer. Rev. Respir. Dis. 118:287-294 (1978)), viral infections (Empey, D.W. et al., Amer. Rev. Respir. Dis. 113:131-139 (1976)) and certain occupational and chemicals (Chan-Yeung, M. et al., Amer. Rev. Respir. Dis. 113:131-139 (1976)) and certain occupational and chemicals (Chan-Yeung, M. et al., Amer. J. Med. 72:411-415 (1982), Durham, S.R. et al., J. Allergy Clin. Immunol. 79:398-406 (1987), Lam, S. et al., J. Allergy Clin. Immunol. 72:134-139 (1983), Lam, S. et al., J. Allergy Clin. Immunol. 63:28-34 (1979)).

50 In fact, there is evidence to suggest that airway hyperresponsiveness is a consequence rather than a predisposing factor of asthma (Boulet, L-P. et al., J. Allergy. Clin. Immunol. 71:399-406 (1983), Chan-Yeung, M. et al., Amer. J. Med. 72:411-415 (1982), Gundel, R.H. et al., Amer. Rev. Respir. Dis. 131:875-879 (1985), Empey, D.W. et al., Amer. Rev. Respir. Dis. 130:56-58 (1984)), air pollutants (Golden, J.A. et al., Amer. Rev. Respir. Dis. 118:287-294 (1978)), viral infections (Empey, D.W. et al., Amer. Rev. Respir. Dis. 113:131-139 (1976)) and certain occupational and chemicals (Chan-Yeung, M. et al., Amer. Rev. Respir. Dis. 113:131-139 (1976)) and certain occupational and chemicals (Chan-Yeung, M. et al., Amer. J. Med. 72:411-415 (1982), Durham, S.R. et al., J. Allergy Clin. Immunol. 79:398-406 (1987), Lam, S. et al., J. Allergy Clin. Immunol. 72:134-139 (1983), Lam, S. et al., J. Allergy Clin. Immunol. 63:28-34 (1979)).

113:131-139 (1976), Lam S. et al., *J. Allergy Clin. Immunol.* 63:28-34 (1979)).

5 While the mechanisms underlying the pathogenesis of airway hyperresponsiveness are not known, results from many studies suggest, as indicated above, that eosinophil infiltration and desquamation of the bronchial epithelium are involved (DeMonchy, J.G.R. et al., *Am. Rev. Respir. Dis.* 131:373 (1985); Laitinen, L.A. et al., *Am. Rev. Respir. Dis.* 137:62 (1988)). Since eosinophil mediators have been shown to damage airway epithelial cells *in vitro* these two events may be linked (Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527 (1986)).

10 **B. Asthma and Intercellular Adhesion**

1 The present invention derives, in part, from the development of an assay capable of identifying agents having therapeutic potential in the treatment of asthma.

15 As used herein, "asthma" refers to either allergic or idiosyncratic asthma. An agent is said to have a therapeutic potential in the treatment of asthma if it may lessen (i.e. attenuate) the severity, extent or duration of the asthma symptoms. Such agents are preferably identified through the use of the following "asthma model system". As used herein, an agent is said to be able to treat asthma if, when administered to a patient, the agent is capable of attenuating either the severity, extent or duration of the asthma symptoms.

20 One aspect of the present invention derives from the discovery that the migration of eosinophils into the lung is dependent upon intercellular adhesion, and specifically that such adhesion is dependent upon an "ICAM-1" ("Intercellular Adhesion Molecule-1") interaction.

25 As used herein, a molecule is a member of the CD18 family of glycoproteins if it contains either an alpha subunit of a member of the CD18 family of glycoproteins (i.e. a CD11 subunit), a beta subunit of a member of the CD18 family of glycoproteins (i.e. a CD18 beta subunit), or both an alpha and a beta subunit of a member of the CD18 family of glycoproteins. Thus, as used herein, a member of the CD18 family of glycoproteins includes molecules having only one subunit of a CD18 member as well as heterodimer (i.e. a molecule having both an alpha and a beta subunit of a member of the CD18 family. All such molecules may be either bound to a membrane or solid support or unbound (i.e. "soluble").

30 "ICAM-1" is the natural ligand for the CD18 family of glycoprotein receptor molecules (Rothlein, R. et al., *J. Immunol.* 137:1270 (1986); Marlin, S.D. et al., *Cell* 51:813 (1987)). ICAM-1 is a 76-97 Kd glycoprotein. ICAM-1 is not a heterodimer. The identification, characterization, and amino acid sequence of ICAM-1, and the production of antibody reactive with ICAM-1 and other adhesion molecules are disclosed in European Patent Application Serial No. 289,949 (which reference is incorporated herein by reference) and in Rothlein, R. et al. (*J. Immunol.* 137:1270-1274 (1986)), Smith, G.W. et al., in *Structure and Function of Molecules Involved in Leukocyte Adhesion*, A.S. Rosenthal, et al., Eds. (Springer-Verlag, New York, 1989); Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988) and Barton, R.W. et al., *J. Immunol.* 143, (1989)), all of which references are incorporated herein by reference).

35 In brief, ICAM-1 is a cell surface glycoprotein expressed on non-hematopoietic cells such as vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, and fibroblasts, and on hematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal centered B cells and dendritic cells in tonsils, lymph nodes, and Peyer's patches. ICAM-1 is highly expressed on vascular and dendritic cells in tonsils, lymph nodes, and tonsils showing reactive hyperplasia. ICAM-1 is expressed in low amounts on peripheral blood lymphocytes. ICAM-1 appears to be required for neutrophil migration into inflamed tissues. Phorbol ester-stimulated differentiation of some myelomonocytic cell lines greatly increases ICAM-1 expression. Thus, ICAM-1 is preferentially expressed at sites of inflammation, and is not generally expressed by quiescent cells. ICAM-1 expression on dermal fibroblasts is increased threefold to fivefold by either interleukin 1 or gamma interferon at levels of 10 U/ml over a period of 4 or 10 hours, respectively. The induction is dependent on protein and mRNA synthesis and is reversible.

40 ICAM-1 displays molecular weight heterogeneity in different cell types with a molecular weight of 97 kd on fibroblasts, 114 kd on the myelomonocytic cell line U937, and 90 kd on the B lymphoblastoid cell JY. ICAM-1 biosynthesis has been found to involve an approximately 73 kd intracellular precursor. The non-N-glycosylated form resulting from tunicamycin treatment (which inhibits glycosylation) has a molecular weight of 55 kd.

45 ICAM-1 isolated from phorbol ester stimulated U937 cells or from fibroblast cells yields an identical major product having a molecular weight of 60 kd after chemical deglycosylation. ICAM-1 monoclonal antibodies interfere with the adhesion of phytohemagglutinin blasts to LFA-1 deficient cell lines. Pretreatment of fibroblasts, but not lymphocytes, with monoclonal antibodies capable of binding ICAM-1 inhibits

lymphocyte-fibroblast adhesion. Pretreatment of lymphocytes, but not fibroblasts, with antibodies against LFA-1 has also been found to inhibit lymphocyte-fibroblast adhesion.

ICAM-1 is, thus, the binding ligand of molecules of the CD18 family of glycoproteins. It is inducible on fibroblasts and endothelial cells *in vitro* by inflammatory mediators such as IL-1, gamma interferon and tumor necrosis factor in a time frame consistent with the infiltration of lymphocytes into inflammatory lesions *in vivo* (Dustin, M.L., et al., *J. Immunol.* 137:245-254, (1986); Prober, J.S., et al., *J. Immunol.* 137:1893-1896, (1986)). Further ICAM-1 is expressed on non-hematopoietic cells such as vascular endothelial cells, thymic epithelial cells, other epithelial cells, and fibroblasts and on hematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal center B-cells and dendritic cells in tonsils, lymph nodes and Peyer's patches (Dustin, M.L., et al., *J. Immunol.* 137:245-254, (1986)). ICAM-1 is expressed on keratinocytes in benign inflammatory lesions such as allergic eczema, lichen planus, exanthema, urticaria and bullous diseases. Allergic skin reactions provoked by the application of a hapten on the skin to which the patient is allergic also revealed a heavy ICAM-1 expression on the keratinocytes. On the other hand toxic patches on the skin did not reveal ICAM-1 expression on the keratinocytes. ICAM-1 is present on keratinocytes from biopsies of skin lesions from various dermatological disorders and ICAM-1 expression is induced on lesions from allergic patch tests while keratinocytes from toxic patch test lesions failed to express ICAM-1.

Yet another aspect of the invention is the discovery that agents which prevent or inhibit cellular adhesion may be employed in the treatment of asthma.

One example of agents which may be used in accordance with the present invention are ICAM-1 and functional derivatives of ICAM-1. Since ICAM-1 mediates cellular adhesion by binding to a receptor molecule on the eosinophil cell surface, functional derivatives of ICAM-1 which can bind to the ICAM-1 receptor present on eosinophils will compete with the ICAM-1 on lung endothelial cells, thus attenuating cellular adhesion of the eosinophils, and providing a treatment for asthma.

A "functional derivative" of ICAM-1 is a compound which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of ICAM-1. The term "functional derivatives" is intended to include the "fragments," "variants," "analog," or "chemical derivatives" of a molecule. A "fragment" of a molecule such as ICAM-1, is meant to refer to any polypeptide subset of the molecule. Fragments of ICAM-1 which have ICAM-1 activity and which are soluble (i.e not membrane bound) are especially preferred.

ICAM-1 is composed of 7 domains (Staunton, D.E. et al., *Immunol. Today* 9:213-215 (1988); Staunton, D.E. et al., *Cell* 52:925-934 (1988); Staunton, D.E. et al., *Cell* 56:849-854 (1989); Staunton, D.E. et al., *Tissue Antigens* 33:287 (1989), all of which references are incorporated herein by reference). The domains of ICAM-1 are shown in Figure 2. Domains 1 and 2 have been found to be important for the binding of ICAM-1 to its receptor molecule (Staunton, D.E. et al., *Tissue Antigens* 33:286 (1989); Staunton, D.E. et al., *FASEB J.* 3:A448 (1989) both of which references are incorporated herein by reference). In accordance with the present invention, ICAM-1 functional derivatives, and especially such derivatives which comprise fragments or mutant variants of ICAM-1 which possess both domains 1 and 2 can be used in the treatment or therapy of asthma. More preferred for such treatment or therapy are ICAM-1 fragments or mutant variants which contain domain 2 of ICAM-1. Most preferred for such treatment or therapy are ICAM-1 fragments or mutant variants which contain domain 1 of ICAM-1.

A "variant" of a molecule such as ICAM-1 is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules not found in the other, or if the sequence of amino acid residues is not identical. An "analog" of a molecule such as ICAM-1 is meant to refer to a molecule substantially similar in function to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). "Toxin-derivatized" molecules constitute a special class of "chemical derivatives." A "toxin-derivatized" molecule is a molecule (such as ICAM-1 or an antibody) which contains a toxin moiety. The binding of such a molecule to a cell brings the toxin moiety into close proximity with the cell and thereby promotes cell death. Any suitable toxin moiety may be employed; however, it is preferable to employ toxins such as, for example, the ricin toxin, the diphtheria toxin, radioisotopic toxins, membrane-

channel-forming toxins, etc. Procedures for coupling such moieties to a molecule are well known in the art.

An additional example of agents which may be used in accordance with the present invention to treat asthma are LFA-1, Mac-1 or p150,95, or the functional derivatives of these molecules. Such molecules and their functional derivatives can provide a treatment for asthma by virtue of their capacity to bind to the ICAM-1 of endothelial cells; and thus impair the ability of such cells to mediate binding and adhesion of eosinophils.

Of special interest to the present invention are functional derivatives of LFA-1, Mac-1 or p150,95 which are soluble molecules. Of special interest are functional derivatives of these molecules which are heterodimers (containing both the alpha and beta subunits of the molecules) and monomeric derivatives capable of binding ICAM-1. Soluble heterodimers are especially preferred.

ICAM-1 and the members of the CD18 family of molecules are immunogenic molecules. Thus, it is possible to obtain antibodies capable of binding to ICAM-1 or members of the CD18 family of molecules. Such antibodies may be used in accordance with the methods of the present invention in the treatment of asthma.

Such antibodies may be obtained by introducing either the purified molecules (or cells which naturally express these molecules) into an appropriate animal, as by intraperitoneal injection, etc. If desired, the serum of such an animal may be removed and used as a source of polyclonal antibodies capable of binding these molecules. It is, however, preferable to remove splenocytes from such animals, to fuse such spleen cells with a myeloma cell line and to permit such fusion cells to form a hybridoma cell which secretes monoclonal antibodies capable of binding ICAM-1 or members of the CD18 family of molecules.

The hybridoma cells, obtained in the manner described above may be screened as described above to identify desired hybridoma cells that secrete antibody capable of binding either to ICAM-1 or to members of the CD18 family of molecules (either the alpha or beta subunit).

Since such antibodies have the capacity to bind to ICAM-1 or its receptor, they (and their fragments having antigen binding ability, such as Fab, F(ab)₂, etc.) may be used to attenuate cellular adhesion, and thus provide an additional example of an agent which may be used in accordance with the present invention to treat asthma.

As indicated above, both polyclonal and monoclonal antibodies may be employed in accordance with the present invention. Of special interest to the present invention are antibodies to ICAM-1 (or their functional derivatives), or to members of the CD18 family (or their functional derivatives), which are produced in humans, or are "Humanized" (i.e. non-immunogenic in a human) by recombinant or other technology. Humanized antibodies may be produced, for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (i.e. chimeric antibodies) (Robinson, R.R. et al., International Patent Publication PCT/US86/02269; Akira, K. et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison, S.L. et al., European Patent Application 173,494; Neuberger, M.S. et al., PCT Application WO 86/01533; Cabilly, S. et al., European Patent Application 125,023; Better, M. et al., Science 240:1041-1043 (1988); Liu, A.Y. et al., Proc. Natl. Acad. Sci. USA 84:3439-3443 (1987); Liu, A.Y. et al., J. Immunol. 139:3521-3526 (1987); Sun, L.K. et al., Proc. Natl. Acad. Sci. USA 84:214-218 (1987); Nishimura, Y. et al., Canc. Res. 47:999-1005 (1987); Wood, C.R. et al., Nature 314:446-449 (1985); Shaw et al., J. Natl. Cancer Inst. 80:1553-1559 (1988).

General reviews of "humanized" chimeric antibodies are provided by Morrison, S.L. (Science, 229:1202-1207 (1985)) and by Oi, V.T. et al., BioTechniques 4:214 (1986)).

Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (Jones, P.T. et al., Nature 321:552-525 (1986); Verhoeyan et al., Science 239:1534 (1988); Beidler, C.B. et al., J. Immunol. 141:4053-4060 (1988)).

The anti-asthma agents of the present invention may be obtained by natural processes (such as, for example, by inducing an animal, plant, fungi, bacteria, etc., to produce a non-immunoglobulin antagonist of ICAM-1, or by inducing an animal to produce polyclonal antibodies capable of binding to ICAM-1); by synthetic methods (such as, for example, by using the Merrifield method for synthesizing polypeptides to synthesize ICAM-1, functional derivatives of ICAM-1, or protein antagonists of ICAM-1 (either immunoglobulin or non-immunoglobulin)); by hybridoma technology (such as, for example, to produce monoclonal antibodies capable of binding to ICAM-1); or by recombinant technology (such as, for example, to produce the anti-asthma agents of the present invention in diverse hosts (i.e., yeast, bacteria, fungi, cultured mammalian cells, etc.), or from recombinant plasmids or viral vectors), or by proteolysis. The choice of which method to employ will depend upon factors such as convenience, desired yield, etc. It is not necessary to employ only one of the above-described methods, processes, or technologies to produce a particular anti-asthma agent; the above-described processes, methods, and technologies may be combined in order to obtain a particular anti-asthma agent.

Functional derivatives of ICAM-1, or a member of the CD18 family, having up to about 100 residues may be conveniently prepared by in vitro synthesis. If desired, such fragments may be modified by reacting targeted amino acid residues of the purified or crude protein with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The resulting covalent derivatives may be used to identify residues important for biological activity. In the embodiments listed below, this aspect of the invention is described with reference to the functional derivatives of ICAM-1. Such methods may also be employed to produce functional derivatives of any member of the CD18 family of molecules.

Cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives.

Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)-propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful, the reaction is

preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylissurea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues per se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare

labeled proteins for use in radioimmunoassay, the chloramine T method being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($\text{R}'-\text{N}-\text{C}-\text{N}-\text{R}'$) such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia 4,4-dimethylpental) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking an ICAM-1 functional derivative molecule to a water-insoluble support matrix or surface for use in the method for cleaving an ICAM-1 functional derivatives fusion polypeptide to release and recover the cleaved polypeptide. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,018; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Functional derivatives of ICAM-1 having altered amino acid sequences can also be prepared by mutations in the DNA. The nucleotide sequence which encodes the ICAM-1 gene is shown in Figure 1. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown in Figure 1. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity.

Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see EP Patent Application Publication No. 75,444).

At the genetic level, these functional derivatives ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the ICAM-1 molecule, thereby producing DNA encoding the functional derivative, and thereafter expressing the DNA in recombinant cell culture. The functional derivatives typically exhibit the same qualitative biological activity as the naturally occurring analog. They may, however, differ substantially in such characteristics with respect to the normally produced ICAM-1 molecule.

While the site for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed ICAM-1 functional derivatives screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, site-specific mutagenesis.

Preparation of an ICAM-1 functional derivative molecule in accordance herewith is preferably achieved by site-specific mutagenesis of DNA that encodes an earlier prepared functional derivatives or a nonvariant version of the protein. Site-specific mutagenesis allows the production of ICAM-1 functional derivatives through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman et al., DNA 2:183 (1983), the disclosure of which is incorporated herein by reference.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al., Third Cleveland Symposium on Macromolecules and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981), the disclosure of which is incorporated herein by reference. These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira et al., Meth. Enzymol. 153:3 (1987)) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea et al., Proc. Natl. Acad. Sci. (USA) 75:5765 (1978). This primer is then annealed with the single-stranded protein-sequence-containing vector, and subjected to DNA-polymerizing enzymes such as E. coli polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as JM101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated protein region may be removed and placed in an appropriate vector for protein production, generally an expression vector of the type that may be employed for transformation of an appropriate host.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably 1 to 10 residues, and, typically are contiguous.

Amino acid sequence insertions include amino and/or carboxyl-terminal fusions of from one residue to polypeptides of essentially unrestricted length, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the complete ICAM-1 molecule sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5. An example of a terminal insertion includes a fusion of a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus of the molecule to facilitate the secretion of the ICAM-1 functional derivative from recombinant hosts.

The third group of functional derivatives are those in which at least one amino acid residue in the ICAM-1 molecule, and preferably, only one, has been removed and a different residue inserted in its place. Such substitutions preferably are made in accordance with the following Table 1 when it is desired to modulate finely the characteristics of the ICAM-1 molecule.

TABLE 1

Original Residue	Exemplary Substitutions
5	
Ala	gly; ser
Arg	lys
Asn	gln; his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	ala; pro
His	asn; gln
Ile	leu; val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; tyr; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

25

Substantial changes in functional or immunological identity are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, e.g., glutamyl or aspartyl; or (e) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine.

Most deletions and insertions, and substitutions in particular, are not expected to produce radical changes in the characteristics of the ICAM-1 molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a functional derivative typically is made by site-specific mutagenesis of the native ICAM-1 molecule-encoding nucleic acid, expression of the variant nucleic acid in recombinant cell culture, and, optionally, purification from the cell culture, for example, by immunoaffinity adsorption on an anti-ICAM-1 molecule antibody column (to absorb the functional derivative by binding it to at least one remaining immune epitope).

The activity of the cell lysate or purified ICAM-1 molecule functional derivative is then screened in a suitable screening assay for the desired characteristic. For example, a change in the immunological character of the functional derivative, such as affinity for a given antibody, is measured by a competitive type immunoassay. Changes in immunomodulation activity are measured by the appropriate assay. Modifications of such protein properties as redox or thermal stability, biological half-life, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

55 C. Administration of the Compositions of the Present Invention

The therapeutic effects of the anti-asthma agents of the present invention may be obtained by providing such agents to a patient by any suitable means (i.e. intravenously, intramuscularly, subcutaneously,

enterally, or parenterally). It is preferred to administer the agents of the present invention intranasally as by nasal spray, swab, etc. It is especially preferred to administer such agents by oral inhalation, or via an oral spray or oral aerosol. When administering agents by injection, the administration may be by continuous infusion, or by single or multiple boluses.

5 The therapeutic advantages of any of the above-discussed agents can be augmented through the use of functional derivatives possessing additional amino acid residues added to enhance coupling to carrier or to enhance the activity of the agent. The scope of the present invention is further intended to include functional derivatives of ICAM-1 which lack certain amino acid residues, or which contain altered amino acid residues, so long as such derivatives exhibit the capacity to affect cellular adhesion.

10 The antibodies of the present invention and the ICAM-1 molecule and the members of the CD18 family disclosed herein are said to be "substantially free of natural contaminants" if preparations which contain them are substantially free of materials with which these products are normally and naturally found.

15 The present invention extends to the use of antibodies, and biologically active fragments thereof, (whether polyclonal or monoclonal) which are capable of binding to ICAM-1 or to a member of the CD18 family in the treatment of asthma.

20 In providing a patient with antibodies, or fragments thereof, capable of binding to ICAM-1 or to a member of the CD18 family, or when providing ICAM-1 or a member of the CD18 family (or a fragment, variant, or derivative thereof) to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered. The therapeutically effective dose can be lowered by using combinations of the above-described agents (such as, for example, if anti-ICAM-1 antibody is additionally administered with an anti-LFA-1 antibody). As used herein, one compound is said to be additionally administered with a second compound when the administration of the two compounds is in such proximity of time that both compounds can be detected at the same time in the patient's serum.

25 The anti-asthma agents of the present invention are intended to be provided to recipient subjects in an amount sufficient to lessen or attenuate the severity, extent or duration of the asthma symptoms.

30 The antibody agents of the invention, or their fragments, may be administered either alone or in combination with one or more additional anti-asthma agents (such as methylxanthines (such as theophylline), beta-adrenergic agonists (such as catecholamines, resorcinols, saligenins, and ephedrine), glucocorticoids (such as hydrocortisone), chromones (such as cromolyn sodium) and anticholinergics (such as atropine), in order to decrease the amount of such agents needed to treat the asthma symptoms.

35 The administration of the agent(s) of the invention may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the agent(s) are provided in advance of any asthma symptom. The prophylactic administration of the agent(s) serves to prevent or attenuate any subsequent asthmatic response. When provided therapeutically, the agent(s) are provided at (or shortly after) the onset of a response. The therapeutic administration of the compound(s) serves to attenuate any actual symptom of asthma. The agents of the present invention may, thus, be provided either prior to the onset of an anticipated asthmatic episode (so as to attenuate the anticipated severity, duration or extent of the 40 episode) or after the initiation of the episode.

45 A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

50 The agents of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences (16th ed., Osol, A., Ed., Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of anti-ICAM antibody or ICAM-1 molecule, or their functional derivatives, together with a suitable amount of carrier vehicle.

55 Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb anti-ICAM-1 antibody or ICAM-1, or their functional derivatives. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine, sulfate) and the concentration of macromolecules as

well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate anti-ICAM-1 antibody or ICAM-1 molecules, or their functional derivatives, into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxyethylcellulose or gelatine-microcapsules and poly(methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

15

EXAMPLE 1

LUNG EOSINOPHIL ADHESION REACTIONS DEPEND ON CD11 AND ICAM-1 CELL ADHESION MOLECULES

In order to evaluate the requirement for cell adhesion of eosinophils in their migratory and cytotoxic function, eosinophil adhesion *in vitro* was tested. In particular, the role of the CD18 family of cellular adhesion molecules, and of ICAM-1, in primate eosinophil adhesion to protein coated plastic and to human endothelium was examined.

Eosinophils were obtained by bronchoalveolar lavage from adult male cynomolgus monkeys (*Macaca fascicularis*), purified (>93% by morphology) on a Percoll continuous density gradient, washed, and added to 96 well flat-bottom tissue culture plates at a concentration of 5×10^3 cells/well. After a 60 min. incubation at 37°C, the non-adherent cells were removed using a plate washer. Adherent cells were enumerated visually (no aggregation or degranulation was observed) and by a colorimetric assay which measures eosinophil peroxidase (EPO).

The eosinophils spontaneously adhered and spread on the bottom of untreated wells or of wells coated with immune complexes (made from *Ascaris* extract and serum from an *Ascaris*-immune monkey). In contrast, eosinophils did not adhere well to wells coated with proteins, including bovine serum albumin, normal monkey serum, or *Ascaris* extract. Of various soluble stimuli tested, Platelet Activating Factor (PAF) induced the most pronounced and consistent adherence of eosinophils to protein-coated wells (Figure 3).

The role of CD18 adhesion molecules and of ICAM-1 in this adherence process was tested using monoclonal antibodies reactive with each of these membrane glycoproteins. Antibodies used included: R3.3 and R15.7 (anti-CD18); R3.1 (anti-CD11a); M1/70 and LM2/1 (anti-CD11b); RR2/1 and R6.5.D6 (anti-ICAM-1); and W6/32 (anti HL-A class I).

Adhesion of the eosinophils to immune complex-coated wells appeared to be CD11b-dependent, since mAbs against CD11b and CD18 inhibited adhesion while mAbs against CD11a, ICAM-1, and HL-A did not (Figure 4). Identical results were obtained using eosinophils activated by soluble stimuli, including PAF, and protein-coated wells.

Adhesion of eosinophils to endothelial cells activated by LPS (10 ng/ml) and fixed with glutaraldehyde was also tested. Human umbilical vein endothelial cells were prepared as described by Smith, C.W. et al. (J. Clin. Invest. 82:1746 (1988) which reference is incorporated herein by reference). Adhesion of eosinophils induced by PAF (10^{-7} M) to such activated endothelial cells was partially inhibited by mAbs against CD11a, CD11b, and ICAM, completely inhibited by anti-CD18, and not inhibited by anti-HL-A (Figure 5). These experiments revealed that the observed adhesion was partially dependent on CD11a, CD11b, and ICAM-1.

In summary, primate lung eosinophils behave nearly identically to human neutrophils in membrane adhesion reactions involving CD11a, CD11b, and ICAM-1. The CD18 family of CAMs therefore plays a major role in eosinophil adherence and is responsible to selective tissue accumulation of eosinophils in LAD patients.

EXAMPLE 2

5

ROLE OF ICAM-1 IN EOSINOPHIL INFILTRATION

In order to demonstrate that ICAM-1 contributed to the eosinophil infiltration, airway epithelium desquamation and increased airway responsiveness that characterize the airway inflammation underlying bronchial asthma, the effect of monoclonal antibodies reactive with ICAM-1 on asthma in primates was investigated. Specifically, (a) the contribution of ICAM-1 to eosinophil adhesion to vascular endothelium *in vitro*, (b) the induction of ICAM-1 on airway epithelium *in vitro* and *in vivo* as well as on bronchial vascular endothelium *in vivo*, and (c) the contribution of ICAM-1 to the eosinophil infiltration and increase in airway responsiveness induced by multiple inhalations of antigen *in vivo* were investigated.

Primate lung eosinophils were stimulated with platelet-activating factor (PAF, 10^{-7} M), and incubated in the presence of lipopolysaccharide (LPS, 10 ng/ml) stimulated cultured human umbilical vein endothelial cells (HUVECs) in order to assay for cellular adhesion. Eosinophils were obtained by bronchoalveolar lavage from adult male cynomolgus monkeys (*Macaca fascicularis*) with airway eosinophilia, purified (morphologically >95% pure) on a Percoll continuous density gradient (Ridings, G.A. et al., *J. Immunol. Meth.* 46:113 (1981)), washed and added to 96 well flat bottom tissue culture plates (5×10^5 cells/well). After a 60 minute incubation at 37°C, the non-adherent cells were removed by an automated plate washer. Adherent cells were quantitated visually and by a colorimetric assay for eosinophil peroxidase, EPO (Strath, M. et al., *J. Immunol. Meth.* 83:209 (1985)). Human umbilical vein endothelial cells were isolated, cultured to a confluent monolayer in each well, stimulated for 4 hours with LPS and finally fixed in 1% glutaraldehyde (Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988)). Immune complex wells were made by coating with *Ascaris* extract followed by serum from an *Ascaris*-sensitive monkey.

Adhesion was found to be significantly inhibited by the anti-ICAM-1 monoclonal antibody RR1/1 (Rothlein, R. et al., *J. Immunol.* 137:1270 (1986); Marlin, S.D. et al., *Cell* 51:813 (1987)) (Figure 6A). In contrast, the anti-HLA class 1 control monoclonal antibody W6/32, which also binds to HUVECs (Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988)), did not inhibit eosinophil adherence (Figure 6A).

Adherence of primate lung eosinophils to immune complex coated plastic was not inhibited by RR1/1 (Figure 6B) demonstrating the specificity of the inhibition of adherence to endothelium. These results indicate that ICAM-1 is important for eosinophil adhesion to endothelium and thus may contribute to eosinophil migration into inflamed tissues *in vivo*.

In addition to being required for migration into tissue, leukocyte adhesion is a prerequisite for cytotoxic tissue injury. Inhibition of adhesion of effector to target cells has been reported to reduce both lymphocyte and granulocyte mediated killing *in vitro* and allograft rejection *in vivo* (Martz, E. et al., *J. Immunol.* 133:2972 (1984)). Since eosinophils and their products have been implicated in airway epithelium desquamation (Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527 (1986)) which in turn is strongly linked with airway hyperresponsiveness (Laitinen, L.A. et al., *Am. Rev. Respir. Dis.* 137:62 (1988)) and asthma symptoms (Hargreave, F.E. et al., *J. Allergy Clin. Immunol.* 68:347 (1981); Boulet, L-P et al., *J. Allergy Clin. Immunol.* 71:399 (1983); Chan-Yeung, N. et al., *Am. J. Med.* 72:411 (1982); Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527 (1986)), the effects of various pro-inflammatory cytokines on ICAM-1 induction on airway epithelial cells *in vitro* was investigated.

Using an ELISA assay (Rothlein R. et al., *J. Immunol.* 141:1665 (1988)) and the anti-ICAM-1 monoclonal antibodies RR1/1 and R6.5 (Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988)) it was found that 16 hour stimulation with interleukin-1 bet (IL-1b), human recombinant tumor necrosis factor alpha (TNFa), and human recombinant interferon gamma (IFNg) enhanced ICAM-1 expression on a monolayer of cultured monkey bronchus epithelial cells (Table 2).

Table 2 shows the effects of pro-inflammatory cytokines on induction of ICAM-1 on bronchial epithelial cells *in vitro*. The rhesus monkey bronchus epithelial cell line 4MBr-5 (obtained from American Type Culture Collection) was cultured to a confluent monolayer and then stimulated for 16 hours with IL-1b, TNFa or IFNg. ELISA assays for ICAM-1 [monoclonal antibodies RR1/1 (Rothlein, R. et al., *J. Immunol.* 137:1270 (1986); Marlin, S.D. et al., *Cell* 51:813 (1987)) and R6.5 (Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988))] expression and LFA-1 alpha [monoclonal antibody R3.1 (Rothlein, R. et al., *J. Immunol.* 141:1665 (1988))] expression were performed as previously described (Rothlein, R. et al., *J. Immunol.* 141:1665 (1988)). The numbers represent the mean of optical density units (relative to normal mouse gamma globulin background) for duplicate cultures and is representative of four individual experiments.

TABLE 2

Stimulus	Concentration	Optical Density Units		
		RR1/1 (anti-ICAM-1)	R6.5 (anti-ICAM-1)	R3.1 (anti-LFA-1a)
none	—	92	206	-27
IL-1b	0.01 units/ml	130	253	-33
	1 unit /ml	166	253	-32
	10 units/ml	149	322	-37
	1 unit /ml	100	236	-30
	10 units/ml	131	266	-29
	100 units/ml	159	346	-31
	1000 units/ml	178	416	-36
IFNg	0.01 units/ml	138	276	-16
	1 unit /ml	263	423	-17
	10 units/ml	413	673	-26
	100 units/ml	576	940	-27

The time course of enhanced ICAM-1 expression (Table 3) was found to be similar to that previously reported for HUVECs *in vitro* (Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988)) and human skin keratinocytes *in vivo* (Wantzin, G.L. et al., *J. Am. Acad. Dermatol.* 20:782 (1989)). As expected, the anti-LFA-1 alpha monoclonal antibody R3.1 (Rothlein, R. et al., *J. Immunol.* 141:1665 (1988)) did not bind to unstimulated or stimulated bronchial epithelium (Table 2). These results suggest that ICAM-1 could contribute to leukocyte (e.g., eosinophil) mediated desquamation of airway epithelium *in vivo*.

Table 3 shows the time course for the induction of ICAM-1 on bronchial epithelial cells *in vitro*. The rhesus monkey bronchus epithelial cell line 4MBr-5 (obtained from American Type Culture Collection) was cultured to a confluent monolayer and then stimulated for various time periods with IL-1b (10 ng/ml) or IFNg (10 units/ml). An ELISA assay for ICAM-1 expression [monoclonal antibody R6.5 (Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988))] was performed as previously described (Rothlein, R. et al., *J. Immunol.* 141:1665 (1988)). The numbers represent the mean of optical density units (relative to normal mouse gamma globulin background) for triplicate cultures and is representative of two individual experiments.

TABLE 3

Stimulation Time (hours)	Stimulus	
	IL-1b	IFNg
0	215	215
2	240	250
4	296	349
8	384	349
16	417	828
24	369	812
48	472	672

50

EXAMPLE 3

55

CONTRIBUTION OF ICAM-1 TO DESQUAMATION *IN VIVO*

To further investigate the ability of ICAM-1 to contribute to eosinophil-mediated desquamation of airway epithelium *in vivo*, immunohistochemical staining was conducted to determine if multiple inhalations of antigen induce ICAM-1 expression on airway epithelium *in vivo*.

Tissues were stained using a modification of a previously described protocol (Wantzin, G.L. et al., J. Am. Acad. Dermatol. 20:782 (1989)). Briefly, tissue specimens were removed and frozen in liquid nitrogen. After cryo-sectioning, 5-10 micron sections were fixed in acetone for 10 minutes and either stained immediately or stored at -20°C. Staining was performed using the Biotin-Strept Avidin System kit according to manufacturer's protocols (BioGenex, CA). Primarily antibody was incubated with tissue as undiluted culture supernatants (RPMI 1640 medium with 10% FBS) for one hour at room temperature. Blocking for non-specific protein binding was accomplished by applying normal goat serum. AEC (3-amino-9-ethylcarbazole) was used as a substrate and the sections were counterstained with Mayer's Hematoxylin.

Intense staining for ICAM-1 was found on both the epithelium (basilateral portion only) and on the vascular endothelium of a trachea section taken from an *Ascaris* antigen sensitive monkey twenty minutes after the third of three alternate day *Ascaris* inhalations. Staining for LFA-1 alpha (Anderson, D.C. et al., J. Inf. Dis. 152:668 (1985); Anderson, D.C. et al., Ann. Rev. Med. 38:175 (1987); Todd, R.F. et al., Hematol./Oncol. Clinics N. Amer. 2:13 (1988)) but not on airway epithelium (Table 2) revealed a leukocyte infiltration in the interstitium that appeared to be most marked just below the epithelial basement membrane. In addition leukocytes were notable between epithelial cells primarily at the basilateral portion of the epithelium where ICAM-1 staining was most pronounced. Little or no nonspecific staining was observed using mouse serum. Staining (using ICAM-1, LFA-1 alpha and mouse serum) in a trachea section taken from an *Ascaris*-sensitive monkey twenty minutes after a single inhalation of *Ascaris*, revealed, as expected based on the time required for its expression (see Table 3 and references Wantzin, G.L. et al., J. Am. Acad. Dermatol. 20:782 (1989); Smith, C.W. et al., J. Clin. Invest. 82:1748 (1988), which references are incorporated herein by reference), little or no ICAM-1 staining was found on the epithelium or vascular endothelium in this section.

In addition, although pockets of leukocyte infiltration were found, leukocytes were not accumulated just below the epithelial baseline membrane and were not found between epithelial cells. Thus, these immunohistochemical staining results further suggest an ICAM-1 dependent eosinophil-epithelial cell interaction may contribute to the desquamation of airway epithelium found in asthmatics.

30

EXAMPLE 4

35

IN VIVO EFFECT OF ICAM-1 ON ASTHMA

Having demonstrated a potential role for ICAM-1 in the pathogenesis of airway hyperresponsiveness and asthma using the *in vitro* and immunohistochemical protocols described above, the *in vivo* effect of anti-ICAM-1 antibodies was investigated. For these studies, an asthma animal model was employed. The model may be used with any mammal, but it is most preferable to employ primates in the model. To induce an asthmatic episode, inhalations of antigen were provided to a monkey on three alternate days. This regimen induced a consistent (usually greater than 8 fold) increase in airway responsiveness to inhaled methacholine in monkeys (Wegner, C.D. et al., Am. Rev. Respir. Dis. 139:A324 (1989), which reference is incorporated herein by reference). This increase in airway responsiveness is preceded by an intense eosinophil infiltration and is similar in magnitude to that induced in asthmatics during the pollen season (Boulet, L-P et al., J. Allergy Clin. Immunol. 71:399 (1983); Sotomayor, H. et al., Am. Rev. Respir. Dis. 130:56 (1984)) or on continued exposure to occupational allergens (Chan-Yeung, M. et al., Am. J. Med. 72:411 (1982); Lam, S. et al., J. Allergy Clin. Immunol. 72:134 (1983); Lam, S. et al., J. Allergy Clin. Immunol. 63:28 (1979)). In lieu of methacholine, histamine or other similar compounds and methacholine equivalents can be employed.

Using this animal model, the effect of the anti-ICAM-1 monoclonal antibody R6.5 on eosinophil infiltration and the induction of airway hyperresponsiveness *in vivo* was investigated. In accordance with the model, airway cell composition and airway responsiveness were determined three days prior to (Day 0) and three days after (Day 10) three alternate day (Day 3, 5 and 7) inhalations of antigen (Wegner, C.D. et al., Am. Rev. Respir. Dis. 139:A324 (1989)). Airway cell composition was measured by bronchoalveolar lavage (BAL). Airway responsiveness was measured by determining the

concentration (PC₁₀₀) of inhaled methacholine that caused a 100% increase in respiratory system resistance. R6.5 was administered intravenously at 1.76 mg/kg daily on Days 2-9. Studies with R6.5 treatment were compared to bracketing control studies performed on each animal.

R6.5 (anti-ICAM-1) treatment attenuated the eosinophil infiltration in all five animals studies (Figure 7A).

5 The increase in airway responsiveness (decrease in inhaled methacholine PC₁₀₀) was also inhibited in all five animals, markedly in four (Figure 7B). Surprisingly, in two animals (c and d) airway responsiveness not only did not increase (as it had in control studies) but actually decreased (methacholine PC₁₀₀ increased) with R6.5 treatment, despite the multiple inhalations of antigen, demonstrating a reversal of an elevated basal airway responsiveness in these animals.

10 In summary, these results demonstrate that ICAM-1 is selectively induced on chronically inflamed tracheal vascular endothelium *in vivo*, that ICAM-1 contributes to eosinophil adhesion to vascular endothelium *in vitro*, and that an anti-ICAM-1 monoclonal antibody attenuates inhaled antigen-induced eosinophil infiltration *in vivo*. Furthermore, ICAM-1 expression is enhanced on cytokine stimulated airway epithelium *in vitro* and selectively induced on chronically inflamed tracheal epithelium *in vivo* indicating that ICAM-1 may contribute to airway epithelium desquamation *in vivo*. An anti-ICAM-1 monoclonal antibody was able to inhibit the increase in airway responsiveness induced by multiple inhalations of antigen in monkeys.

15 These results indicate that ICAM-1 plays a pivotal role in the pathogenesis of airway hyperresponsiveness and asthma. ICAM-1 similarly contributes to the onset and progression of other diseases characterized by airway inflammation (e.g., chronic bronchitis, emphysema, idiopathic pulmonary fibrosis, etc. (Guenther, C.A. et al., *Am. Rev. Respir. Dis.* 123:79 (1981); Rossi, G.A. et al., *Am. Rev. Respir. Dis.* 129:850 (1984); Hunninghake, G.W. et al., *Am. J. Pathol.* 97:149 (1979); Hunninghake, G.W. et al., *Am. Rev. Respir. Dis.* 123:407 (1981)) or eosinophil infiltration and tissue sensitization / destruction (e.g., rhinitis, nasal polypsis, chronic urticaria and atopic dermatitis (Mygind, N., *Allergy* 34:185 (1979); Mullarkey, M.F. et al., *J. Allergy Clin. Immunol.* 65:122 (1980); Peters, M.S. et al., *J. Invest. Dermatol.* 81:39 (1983); Leiferman, K.M. et al., *N. Engl. J. Med.* 313:282 (1985); Spry, C.J.F. et al., *Int. Archs. Allergy appl. Immun.* 77:252 (1985))). Thus, agents which prevent or attenuate such cellular adhesion may be employed in the treatment of these diseases in the same manner as they may be used to treat asthma.

EXAMPLE 5

30

EFFECTS OF SINGLE AND MULTIPLE INHALATIONS OF ANTIGEN ON AIRWAY RESPONSIVENESS IN MONKEYS

35

In animals (Lanes, S. et al., *J. Appl. physiol.* 61:864-872 (1986), Marsh, W.R. et al., *Amer. Rev. Respir. Dis.* 131:875-879 (1985)), as well as in man (Cartier, A. et al., *J. Allergy Clin. Immunol.* 70:170-177 (1982), Cockcroft, D.W. et al., *Clinical Allergy* 7:503-513 (1977), a single inhalation of an allergen can cause a mild (2-6 fold) increase in airway responsiveness. Repeated allergen exposures, such as those that occur in man during the pollen season (Boulet, L.-P. et al., *J. Allergy Clin. Immunol.* 71:399-406 (1983), Sotomayor, H. et al., *Amer. Rev. Respir. Dis.* 130:56-58 (1984)) or in an occupational setting (Chan-Yeung, M. et al., *Amer. J. Med.* 72:411-415 (1982), Lam, S. et al., *J. Allergy Clin. Immunol.* 72:134-139 (1983), Lam, S. et al., *J. Allergy Clin. Immunol.* 63:28-34 (1979)), have been reported to cause greater (often \pm 10 fold) increases in airway responsiveness.

40 Multiple (4 weekly) instillations of antigen coated beads induce a 10 fold increase in airway responsiveness in monkeys (Gundel, R.H. et al., *Amer. Rev. Respir. Dis.* (1989), which reference is incorporated herein by reference).

45 In order to more develop a useful animal asthma model, the effects of single and multiple antigen inhalations on airway responsiveness to inhaled methacholine in monkeys was investigated. Since airway inflammation (Marsh, W.R. et al., *Amer. Rev. Respir. Dis.* 131:875-879 (1985), Sotomayor, H. et al., *Amer. Rev. Respir. Dis.* 130:56-58 (1984), Lazarus, S., *Amer. J. Med.* 81:2-7 (1986), O'Byrne, P.M., *Chest* 90:575-577 (1986)), especially eosinophil infiltration (Gundel, R.H. et al., *Amer. Rev. Respir. Dis.* (1989), Demonechy, J.G.R. et al., *Amer. Rev. Respir. Dis.* 131:373-375 (1985), Wardlaw, A.J. et al., *Amer. Rev. Respir. Dis.* 137:62-69 (1988)), has been hypothesized to play a role in the pathogenesis of airway hyperresponsiveness, airway cellular composition was concomitantly investigated.

In order to investigate these effects, seven adult male cynomolgus monkeys (*Macaca fascicularis*,

Charles River Primate Imports, Port Washington, NY) weighing 4.6 to 8.2 kg were studied. All animals demonstrated a naturally occurring and reproducible respiratory sensitivity to inhaled Ascaris suum extract. Animals were studied anesthetized with ketamine hydrochloride (1 mg/kg, i.m.; Ketaset, Bristol Laboratories) and xylazine (4 mg/kg, i.m.; Rompun, Miles Laboratories, Inc.), intubated with a cuffed endotracheal tube (5.5 mm ID; Mallinckrodt Critical Care, cat #86048) and seated in an upright position in a specially designed support chair. Ketamine (4 mg/kg, i.m.) was used alone to supplement anesthesia as needed.

10 Airway responsiveness (methacholine PC₁₀₀) followed by airway cell composition (BAL) were determined one day prior to and twenty hours after a single inhalation of Ascaris extract or three days prior to (Day 0) and three days after (Day 10) three alternate day (Days 3, 5 and 7) inhalations of Ascaris extract. All seven animals were studied in both protocols. A letter designation was assigned to each animal for presentation of results.

15 In asthmatics, the peak increase in airway responsiveness occurs three to twenty-four hours post allergen challenge (Cartier, A. et al., *J. Allergy Clin. Immunol.* 70:170-177 (1982), Cockcroft, D.W. et al., *Clinical Allergy* 7:503-513 (1977), Durham, S.R. et al., *J. Allergy Clin. Immunol.* 79:398-406 (1987)). Thus, airway responsiveness was measured twenty hours after the single inhalation of antigen.

20 However, to avoid anesthetizing each animal for a fourth time in a six day interval, airway responsiveness was not measured twenty hours after the multiple inhalation of antigen. Instead, airway responsiveness was measured three days after the multiple inhalations of antigen. It was discovered that airway inflammation (i.e. eosinophil infiltration) still persists three days after antigen inhalation (Wegner, C.D. et al., *Amer. Rev. Respir. Dis.* 135:A221 (1987) which reference is incorporated herein by reference).

25 Ascaris suum extract (Greer Laboratories, cat #B-33) was employed as antigen in the studies. The extract was diluted in phosphate buffered (5mM, pH 7.4) saline (0.5%) (PBS), compressed air nebulized (Bird Micronebulizer, model 8158) and administered by intermittent positive pressure breathing (Bird Mark 7A Respirator) consisting of 30 inhalations to 20 cm H₂O in approximately two minutes. For each animal, the previously determined concentration of Ascaris extract that caused a reproducible 150 to 300% acute increase in respiratory system resistance was used. Methacholine challenges were performed in an identical manner except that they consisted of only 15 breaths in one minute.

30 Respiratory system impedance (Zrs) was measured by discrete frequency (4-40 Hz in 11 equal logarithmic steps) sinusoidal forced oscillations superimposed on tidal breathing as described by Wegner, C.D. et al. (*Respir. Physiol.* 55:47-81 (1983) which reference is incorporated herein by reference). The mean of the real or in-phase component of Zrs over the frequency range was then computed to provide a single value representation of respiratory system resistance (Rrs). Rrs was measured at 3, 7, 10, 15, 20 and 30 minutes after each antigen challenge and at 1 and 3 minutes after each methacholine challenge.

35 Airway Responsiveness was assessed by determining the concentration of nebulized and inhaled methacholine that induced a 100% increase in Rrs (PC₁₀₀). This was accomplished by administering increasing concentrations of methacholine (diluted with PBS) in half-logarithmic steps (at 7 minute intervals) until a greater than 100% increase in Rrs from baseline was obtained. The PC₁₀₀ was then calculated by linear regression analysis of the last two or three points on the logarithm methacholine concentration versus percent increase in Rrs plot.

40 Airway cell composition was assayed by bronchoalveolar lavage (BAL). A pediatric fiberoptic bronchoscope (Olympus Corporation, model BF-3C4) was guided past the carina until it wedged into typically a fifth to seventh generation bronchus. A single 15 ml aliquot of bicarbonate buffered (0.5 mM) normal saline (pH 7.4) was then infused and gently aspirated (return volume range from 7 to 10 ml) through a channel in the bronchoscope. Total leukocytes/ml of BAL was determined using a Coulter counter (Coulter Electronics, model Z_{BI}). Differential cell counts (a total of 200 cells counted) were performed on Wright-Giemsa stained cytocentrifuge (Shandon Cytospin, model 2) preparations. To prevent the effects of a prior BAL on subsequent BAL determinations, the BALs performed prior to and after antigen challenge were done on opposite sides of the lungs.

45 Analysis of variance was performed using the nonparametric Kruskal-Wallis Test (Chi-square approximation). Correlations were performed using Pearson product-moment and Spearman ranks of the variables. A p value of > 0.05 was considered not significant.

50 The single inhalation of antigen caused an acute increase in Rrs (307 ± 62%) that peaked 10 - 15 minutes post challenge, an increase in airway leukocytes (267 ± 19 to 694 ± 142 × 10³/ml of BAL, p > X² = 0.018) and a decrease in methacholine PC₁₀₀ in 3 animals that was moderate (>8 fold) in two (Figure 8A). The increase in airway leukocytes consisted of an infiltration of eosinophils in all animals (Figure 8B), 55 neutrophils in 3 animals (Figure 8C), macrophages/monocytes in 5 animals (Figure 8D) and lymphocytes in 4 animals (Figure 8E). The magnitude and direction of the change in airway responsiveness (methacholine PC₁₀₀) did not correlate with the intensity of the acute increase in Rrs or with the magnitude/existence of

the infiltration of any leukocyte subtype (Table 4).

The multiple inhalations of antigen caused acute increases in Rrs ($178 \pm 48\%$, $380 \pm 83\%$ and $331 \pm 63\%$, respectively), and increase in airway leukocytes (209 ± 42 to $553 \pm 129 \times 10^3/\text{ml}$ of BAL, $p > \chi^2 = 0.0088$) and a decrease in methacholine PC_{100} in all 7 animals that was moderate (> 8 fold) in two and severe (> 80 fold) in three (Figure 9A). The increase in airway leukocytes consisted of a marked infiltration of eosinophils (Figure 9B) and slight infiltration of neutrophils (8.5 ± 2.6 to $31.0 \pm 7.5 \times 10^3/\text{ml}$ of BAL, $p > \chi^2 = 0.010$). There was no significant infiltration of macrophages/monocytes (179 ± 36 to $209 \pm 40 \times 10^3/\text{ml}$ of BAL) or lymphocytes (3.0 ± 1.2 to $3.0 \pm 1.3 \times 10^3/\text{ml}$ of BAL). The magnitude of the increase in airway reactivity (decrease in PC_{100}) did not correlate with the intensity of the eosinophil infiltration or with the magnitude/direction of the change in airway reactivity (PC_{100}) induced by the single inhalation of antigen (Table 5).

TABLE 4

COMPARISON OF CHANGE IN METHACHOLINE PC_{100} TO THE BRONCHOCONSTRICTION AND LEUKOCYTE INFILTRATION INDUCED BY A SINGLE INHALATION OF ANTGEN*						
Animal	Change in Log. PC_{100}	Increase in Rrs (%)	Leukocyte Infiltration ($\times 10^3/\text{ml}$ BAL)			
			Eosin.	Neut.	Mac./Mono.	Lympho.
a	-1.02	494	23	297	105	3.3
b	-0.95	278	266	-33	421	20.5
c	-0.75	181	688	87	104	2.7
d	0.16	244	242	6	253	18.6
e	0.21	525	38	-6	-73	-1.6
f	0.24	68	124	-2	-78	-3.0
g	0.36	360	221	178	264	-0.4.
Mean	-0.25	307	226	76	142	5.8
S.E.	± 0.24	± 63	± 82	± 46	± 69	± 3.7

Definition of abbreviations: Log. - logarithm base 10; PC_{100} - provocative concentration of methacholine required to cause a 100% increase in Rrs; Rrs - respiratory system resistance; BAL - bronchoalveolar lavage; Eosin. - eosin phils; Neut. - neutrophils; Mac./Mono. - macrophages/monocytes; Lympho. - lymphocytes.

* No significant correlations were found.

40

45

50

55

TABLE 5

COMPARISON OF CHANGE IN METHACHOLINE PC₁₀₀ TO EOSINOPHIL INFILTRATION
INDUCED BY MULTIPLE INHALATIONS OF ANTIGEN TO THE CHANGE IN METHACHOLINE
PC₁₀₀ INDUCED BY A SINGLE INHALATION OF ANTIGEN *

15	Animal	Multiple Inhalations of Antigen		Single Inhalation of Ag. Change in Log. PC 100
		Change in Log. PC 100	Eosin. Infiltration ($\times 10^3$ /ml BAL)	
20	d	-2.66	144	0.16
	b	-2.13	352	-0.95
	e	-1.95	409	0.21
	a	-1.07	135	-1.02
	g	-0.97	174	0.36
	c	-0.43	206	-0.75
	f	-0.27	109	0.24
25	Mean	-1.36	218	-0.25
	S.E.	±0.34	±44	±0.24

Definition of abbreviations: Log. = logarithm base 10; PC₁₀₀ = provocative concentration of methacholine required to cause a 100% increase in respiratory system resistance; BAL = bronchoalveolar lavage; Eosin. = eosinophils; Ag. = antigen.

* No significant correlations were found.

35 These experiments demonstrate that non-specific airway hyper-responsiveness, as assessed clinically by responsiveness of the airways to inhaled histamine, methacholine, exercise or cold air, is a characteristic feature of asthma (Boushey, H.A. et al., *Am. Rev. Respir. Dis.* 121:389-413 (1980), Hargrave, F.E. et al., *J. Allergy Clin. Immunol.* 68:347-355 (1981)). While the mechanisms underlying the pathogenesis of airway hyperresponsiveness are not known, results from many studies suggest that leukocyte infiltration (Gundel, 40 R.H. et al., *Amer. Rev. Respir. Dis.* (1989), Marsh, W.R. et al., *Amer. Rev. Respir. Dis.* 131:875-879 (1985), Lazarus, S., *Amer. J. Med.* 81:2-7 (1986), O'Byrne, P.M., *Chest* 90:575-577 (1986), Demonehy J.G.R. et al., *Amer. Rev. Respir. Dis.* 131:373-376 (1985), Wardlaw, A.J. et al., *Amer. Rev. Respir. Dis.* 137:62-69 (1988), *Amer. Rev. Respir. Dis.* 131:373-376 (1985), Wardlaw, A.J. et al., *Amer. Rev. Respir. Dis.* 137:62-69 (1988), Metzger, W.J. et al., *Chest* 89:477-483 (1986)) and/or mediators released by resident or infiltrating cells (Lanes, S. et al., *J. Appl. Physiol.* 61:864-872 (1986), Lazarus, S., *Amer. J. Med.* 81:2-7 (1986) O'Byrne, 45 (Lanes, S. et al., *J. Appl. Physiol.* 61:864-872 (1986), Lazarus, S., *Amer. J. Med.* 81:2-7 (1986) O'Byrne, P.M., *Chest* 90:575-577 (1986), Wardlaw, A.J. et al., *Amer. Rev. Respir. Dis.* 137:62-69 (1988), Aizawa, H. et al., *J. Appl. Physiol.* 59:1918-1923 (1985), O'Byrne, P.M. et al., *Prostaglandins* 4:537-543 (1984), Schulman, E.S., *Chest* 90:578-586 (1986)) are involved.

E.S., Chest 90:578-586 (1986)) are involved. Antigen inhalation is known to induce an acute neutrophil and more chronic eosinophil airway infiltration (Wegner, C.D. et al., Amer. Rev. Respir. Dis. 135:A221 (1987)). A chronic idiopathic airway eosinophilia has been found to be associated with severe (> 80 fold) airway hyperresponsiveness (Wegner, C.D. et al., Amer. Rev. Respir. Dis. 135:A222 (1987)). Multiple (four weekly) intratracheal instillations of antigen-coated beads have been found to induce marked increases in airway eosinophils and responsiveness (> 8 fold) (Gundel, R.H. et al., Amer. Rev. Respir. Dis. (1989)). The experiments presented above provide a comparison of the effects of a single versus multiple (three alternate day) inhalations of *Ascaris suum* extract on airways responsiveness and leukocyte composition in "allergic" monkeys.

The single inhalation of antigen resulted in an acute bronchoconstriction and, as measured 20 hours later, an infiltration of leukocytes (primarily and most consistently eosinophils) as well as an increase in airway responsiveness (decrease in inhaled methacholine PC_{100}) in 3 of 7 animals that was moderate (>8 extract on airways responsiveness and <100 extract on airways responsiveness).

fold) in two. These effects, as well as the frequency which they occurred, are similar to those reported for man. That is, in allergic asthmatics a single inhalation of antigen induces a predominately eosinophilic leukocyte infiltration (DeMonchy, J.G.R. et al., Amer. Rev. Respir. Dis. 131:373-376 (1985)) and an increase (usually mild, <8 fold) in airway responsiveness in some individuals (Cartier, A. et al., J. Allergy Clin. Immunol. 70:170-177 (1982), Cockcroft, D.W. et al., Clinical Allergy 7:503-513 (1977)).

5 In contrast, the multiple inhalations of antigen induced, as assayed three days after the last challenge, an increase in airway responsiveness in all seven monkeys that was moderate (>8 fold) in two and sever (>80 fold) in three. These effects are also consistent with those reported for man. During the pollen season, 10 the airway responsiveness of allergic asthmatics has been reported to increase in all individuals, although to varying degrees (Boulet, L-P. et al., J. Allergy Clin. Immunol. 71:399-406 (1983), Sotomayor, H. et al., Amer. Rev. Respir. Dis. 130:56-58 (1984)). Similarly, repeated exposure to occupational allergens has been shown to increase airway responsiveness in sensitive individuals (Chan-Yeung M., et al., Amer. J. Med. 72:411-415 (1982), Lam, S. et al., J. Allergy Clin. Immunol. 72:134-139 (1983), Lam, S. et al., J. Allergy Clin. Immunol. 63:28-34 (1979)).

15 In both the single and multiple inhalation of antigen protocols, the magnitude/direction of change in airway responsiveness did not correlate with the intensity of the leukocyte infiltration. This finding emphasizes the leukocyte infiltration alone is not enough to infer their direct involvement in the pathogenesis of airway hyperreactivity. Rather, the amount and type (stimulator or inhibitor) of mediators released by the infiltrating and resident cells as well as the interactions occurring between the infiltrating and resident 20 cells (e.g., eosinophils and airway epithelium) are important factors.

25 In summary, as has been previously reported for asthmatics, multiple (but not single) inhalations of antigen induce a consistent (usually > 8 fold) increase in airway responsiveness in monkeys. These findings indicate that this animal model can be used to screen for and identify agents capable of providing a therapy for asthma.

20 While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in 30 the scope of the appended claims.

Claims

- 35 1. The use of an agent selected from
 - (a) an antibody capable of binding to ICAM-1;
 - (b) a fragment of said antibody (a), said fragment being capable of binding to ICAM-1;
 - (c) ICAM-1, being substantially free of natural contaminants;
 - (d) a functional derivative of ICAM-1;
- 40 (e) an antibody capable of binding to a member of the CD18 family of glycoproteins;
 - (f) a fragment of said antibody (e), said fragment being capable of binding to a member of the CD18 family of glycoproteins;
 - (g) a member of the CD18 family of glycoproteins, being substantially free of natural contaminants; and
 - (h) a functional derivative of a member of the CD18 family of glycoproteins.
- 45 2. The method of claim 1 wherein said agent is said antibody (a) capable of binding to ICAM-1, or a fragment of said antibody (a), said fragment being capable of binding to ICAM-1.
3. The method of claim 2 wherein said antibody (a) is a monoclonal antibody.
4. The method of claim 3 wherein said monoclonal antibody is the monoclonal antibody R6.5.
- 50 5. The method of claim 1 wherein said agent is ICAM-1, being substantially free of natural contaminants.
6. The method of claim 1 wherein said agent is a functional derivative of ICAM-1.
7. The functional derivative of the ICAM-1 of claim 6 which contains ICAM-1 domain 1.
8. The functional derivative of the ICAM-1 of claim 6 which contains ICAM-1 domain 2.
- 55 9. The functional derivative of the ICAM-1 of claim 6 which is a soluble derivative of ICAM-1.
10. The method of claim 1 wherein said agent is said antibody (e) capable of binding to a member of the CD18 family of glycoproteins; or a fragment of said antibody (e), said fragment being capable of binding to a member of the CD18 family of glycoproteins.

11. The method of claim 10 wherein said antibody (e) is able to bind to an alpha subunit of said member of the CD18 family of glycoproteins.
12. The method of claim 10 wherein said antibody (e) is able to bind to a beta subunit of said member of the CD18 family of glycoproteins.
- 5 13. The method of claim 1 wherein said agent is a member of the CD18 family of glycoproteins, being substantially free of natural contaminants.
14. The method of claim 13 wherein said member of the CD18 family of glycoproteins contains an alpha subunit of a member of the CD18 family of glycoproteins.
15. The method of claim 13 wherein said member of the CD18 family of glycoproteins contains a beta subunit of a member of the CD18 family of glycoproteins.
- 10 16. The method of claim 13 wherein said member of the CD18 family of glycoproteins is a heterodimer containing both an alpha and a beta subunit of a member of the CD18 family of glycoproteins.
17. The method of claim 13 wherein said agent is a functional derivative of a member of the CD18 family of glycoproteins.
- 15 18. A method of identifying an agent capable of therapeutic potential in the treatment of asthma which comprises administering said agent to a non-human mammal having received multiple inhalations of an antigen.
19. The method of claim 18 wherein any increase in airway responsiveness in said mammal is measured with methacholine or histamine.

20

25

30

35

40

45

50

55

Neu eingereicht / Newly filed
Nouvellement déposé

FIG. 1.

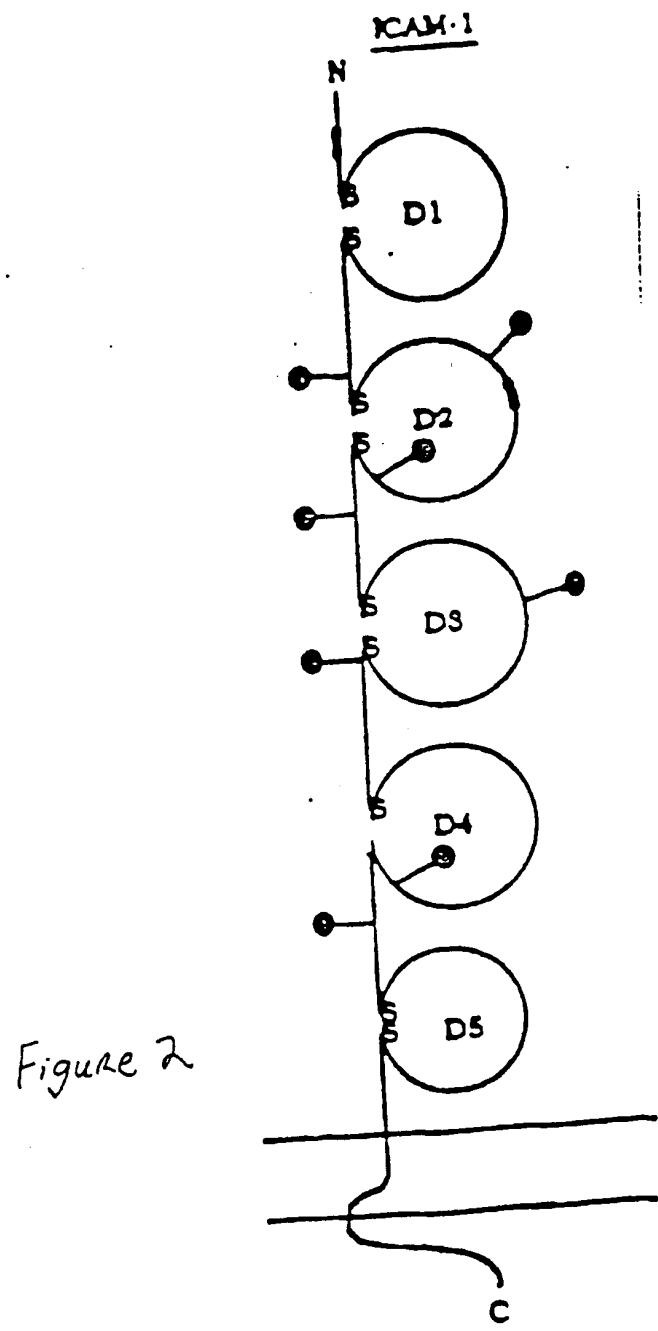


Figure 3

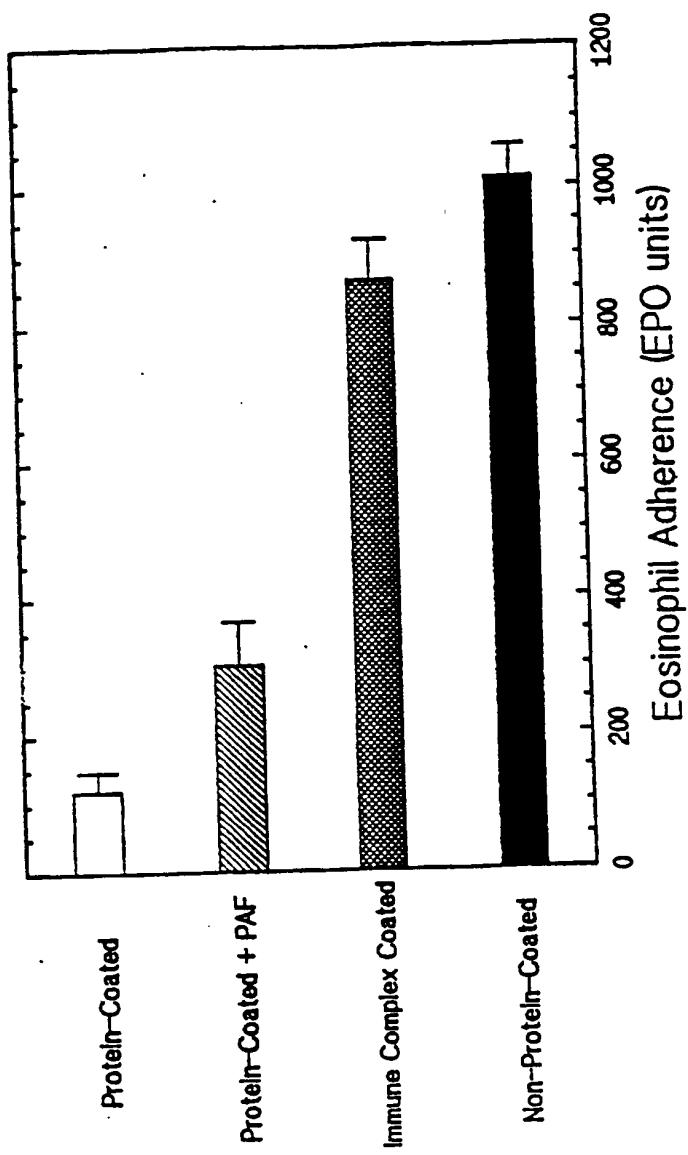


Figure
4

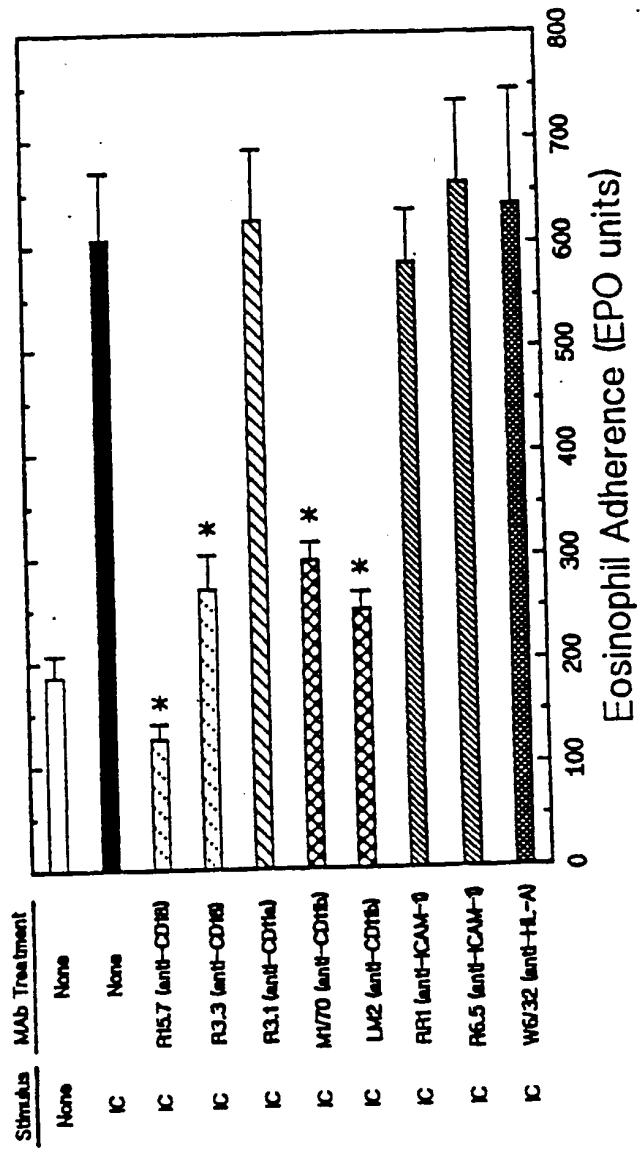
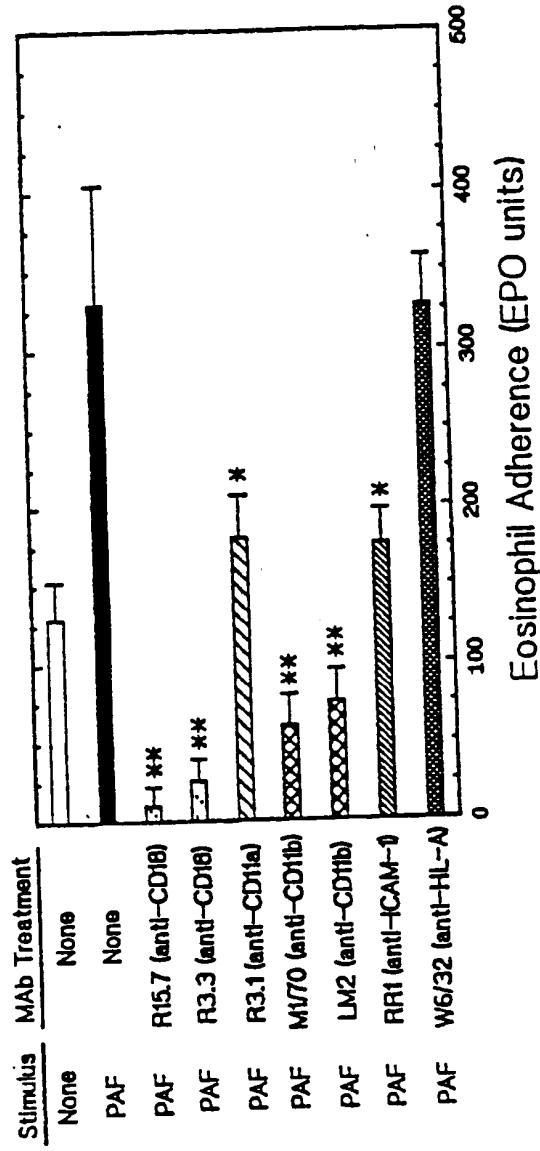


Figure 5



Neu eingereicht / Newly filed
Nouvellement déposé

FIG. 6A

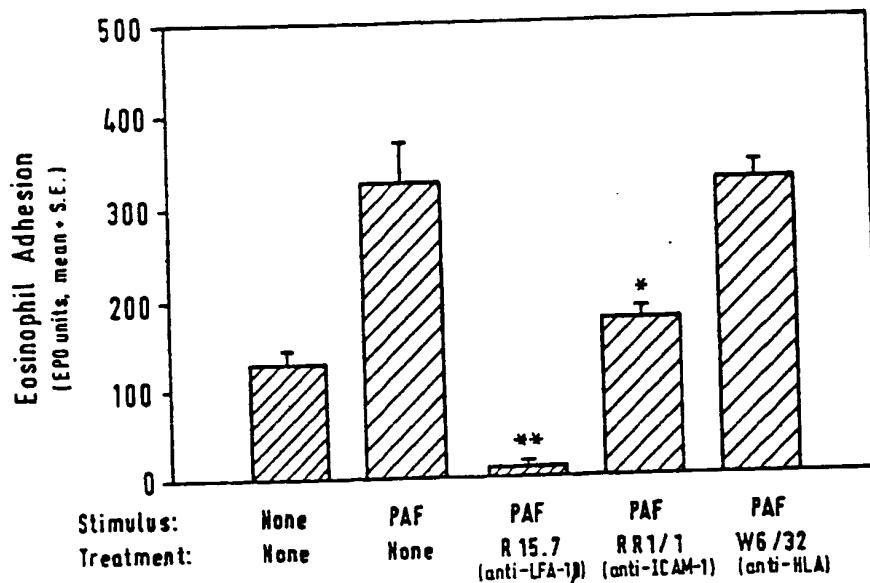


FIG. 6B

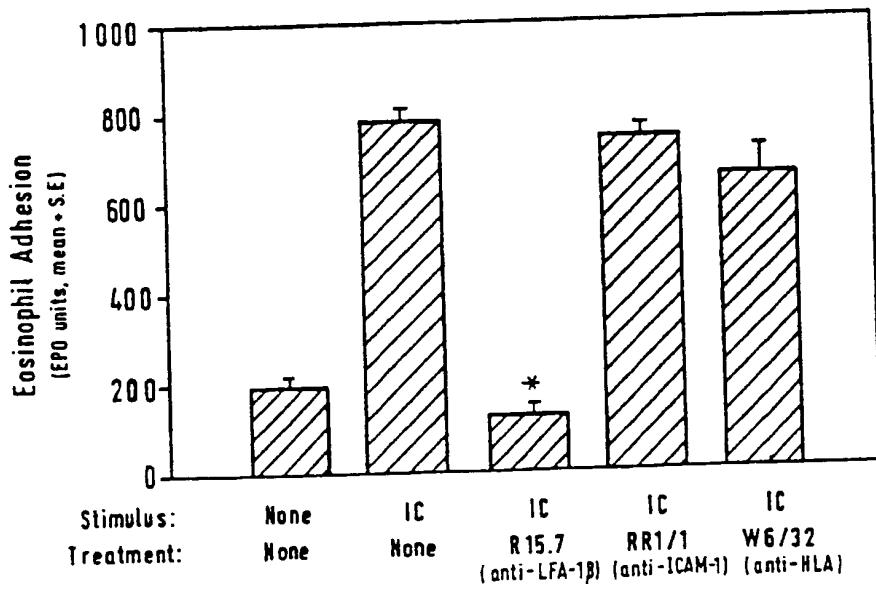
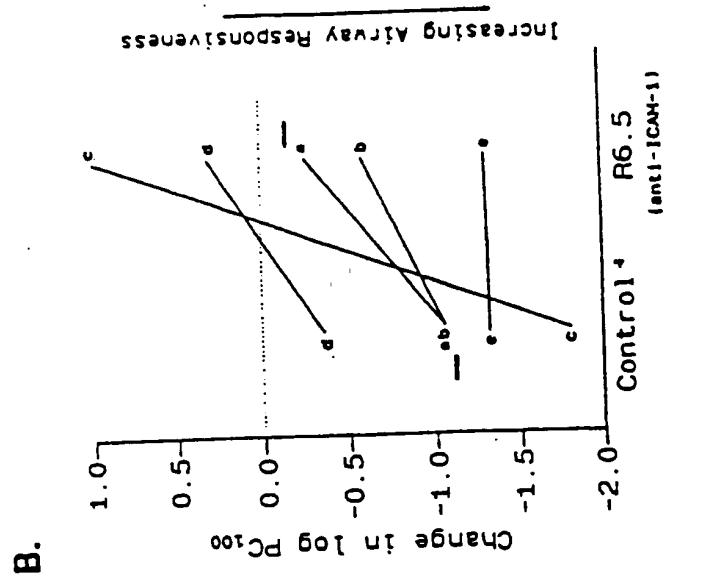
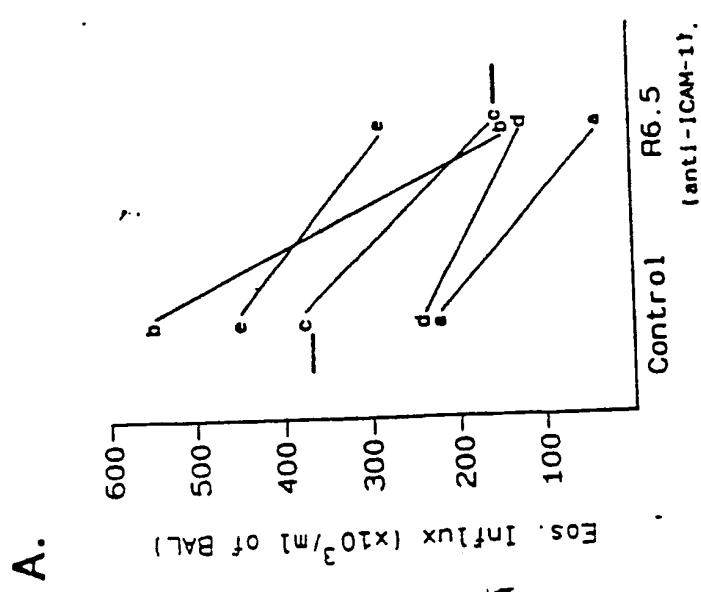


Figure 7.



Increasing Airway Responsiveness

Control • R6.5

(anti-ICAM-1)

Figure 8

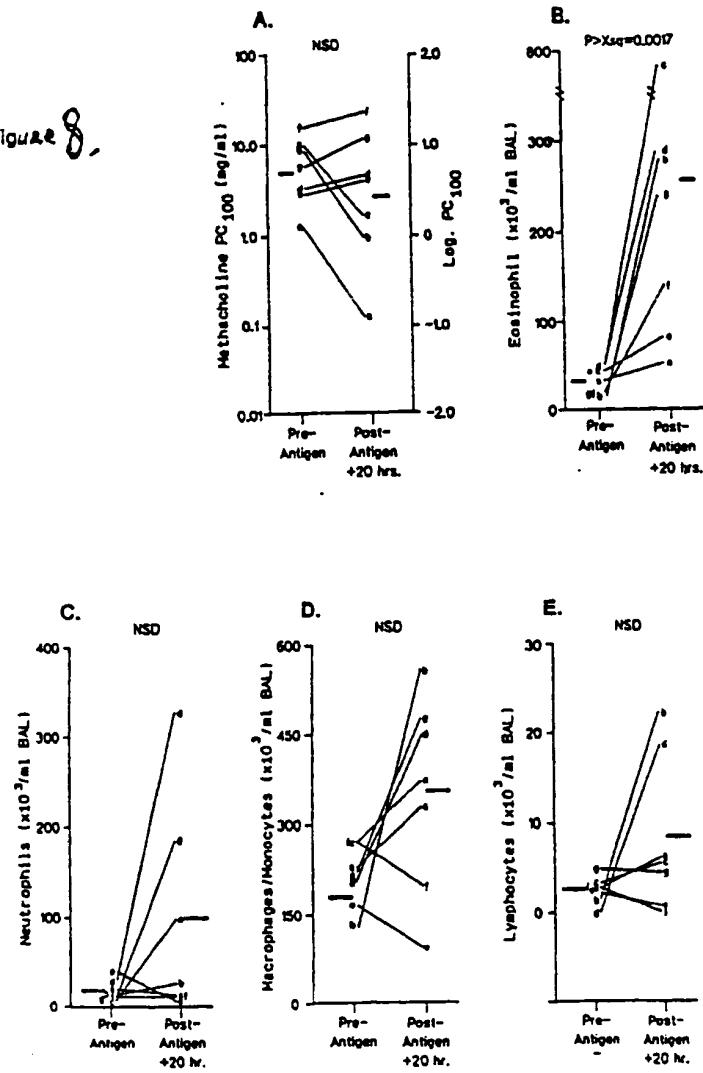
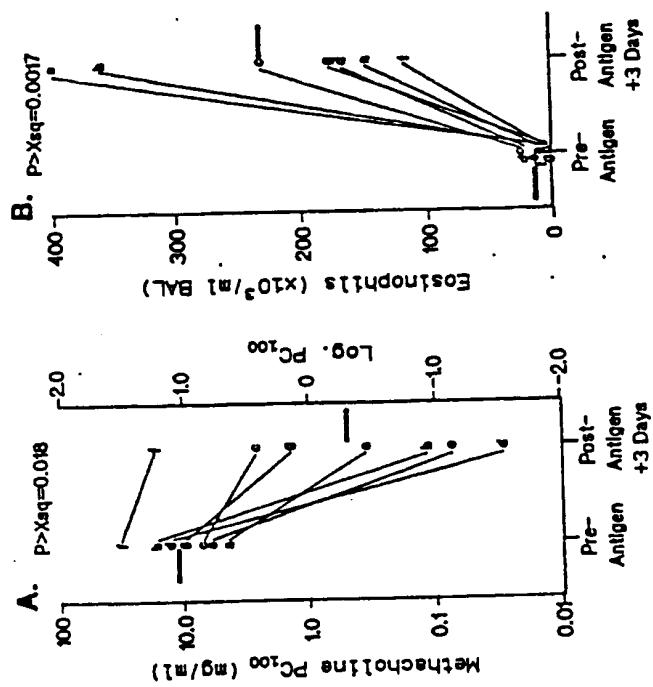


Figure 9





European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT
which under Rule 45 of the European Patent Convention
shall be considered, for the purposes of subsequent
proceedings, as the European search report

Application number

EP 90 10 4423

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	<p>JOURNAL OF IMMUNOLOGY, vol. 140, no. 5, March 1, 1988, pages 1500-1505, The American Association of Immunologists, US; A.M. LAMAS et al.: "Studies on the adhesive interaction between purified human eosinophils and cultured vascular endothelial cells"</p> <p>* Pages 1504, column 1, lines 40-44 *</p> <p>---</p>	1-17	A 61 K 37/02 A 61 K 39/395
A	<p>EP-A-0 289 949 (DANA FARBER CANCER INSTITUTE)</p> <p>* The whole document *</p> <p>---</p>	1-17	C 12 N A 61 K
		./.	
INCOMPLETE SEARCH <p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.</p> <p>Claims searched completely: 1-17</p> <p>Claims searched incompletely: 18,19</p> <p>Claims not searched: 18,19</p> <p>Reason for the limitation of the search: Method for treatment of the human or animal body by surgery or therapy (see Art. 52(4) of the European Patent Convention).</p>			
Place of search		Date of completion of the search	Examiner
The Hague		22-06-1990	VAN PUTTEN
CATEGORY OF CITED DOCUMENTS <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons S : member of the same patent family, corresponding document</p>			



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application number

EP 90 10 4423 -2-

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (IN CL 4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	SCIENCE, vol. 247, January 26, 1990 pages 456-459, Washington D.C., US; G.D. WEGNER et al.: "Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma" * The whole article * --	1-17	
T	EP-A-0 314 863 (BAYLOR COLLEGE OF MEDICINE AND BOEHRINGER INGELHEIM) --		TECHNICAL FIELDS SEARCHED (IN CL 4)
T	EP-A-0 319 815 (MOLECULAR THERA- PEUTICS) -----		



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) Publication number: 0 387 701 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification :
12.08.92 Bulletin 92/33

(51) Int. Cl.⁵: A61K 37/02, A61K 39/395

(21) Application number : 90104423.0

(22) Date of filing : 08.03.90

(54) Use of intercellular adhesion molecules, and their binding ligands in the treatment of asthma.

(30) Priority : 09.03.89 US 321018
09.03.89 US 321239
09.03.89 US 321237
16.03.89 US 324481
01.09.89 US 401409

(43) Date of publication of application :
19.09.90 Bulletin 90/38

(45) Publication of the grant of the patent :
12.08.92 Bulletin 92/33

(64) Designated Contracting States :
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(56) References cited :
EP-A- 0 289 949
EP-A- 0 314 863
EP-A- 0 319 815

(56) References cited :
JOURNAL OF IMMUNOLOGY, vol. 140, no. 5,
March 1, 1988, pages 1500-1505, The American
Association of Immunologists, US;
A.M.LAMAS et al.: "Studies on the adhesive
interaction between purified human eos-
inophils and cultured vascular endothelial
cells" *Pages 1504, column 1, lines 40444 *
SCIENCE, vol. 247, January 26, 1990 pages
456-459, Washington D.C., US; G.D. WEGNER
et al.: "Intercellula adhesion molecule-
1(ICAM-1) in the pathogenesis of asthma"

(73) Proprietor : Boehringer Ingelheim
Pharmaceuticals Inc.
90 East Ridge P.O. Box 368
Ridgefield Connecticut 06877 (US)

(72) Inventor : Wegner, Craig D.
10 Skyview Drive
New Milford Connecticut 06766 (US)
Inventor : Gundel, Robert H.
RR2 Box 74 Hunds Corner Road
Pawling New York 12564 (US)
Inventor : Rothlein, Robert
32 Tammany Trail
Danbury Connecticut 06811 (US)

(74) Representative : Laudien, Dieter, Dr. et al
Boehringer Ingelheim GmbH, Abteilung
Patente
W-6507 Ingelheim am Rhein (DE)

EP 0 387 701 B1

Note : Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

Description

The present invention relates to the use of intercellular adhesion molecules such as ICAM-1 in the treatment of asthma. The invention additionally concerns ligand molecules capable of binding to such intercellular adhesion molecules, and to an assay for detecting agents having therapeutic potential in the treatment of asthma.

5

A. Cellular Adhesion

10

Leukocytes must be able to attach to cellular substrates in order to properly defend the host against foreign invaders such as bacteria or viruses. An excellent review of the defense system is provided by Eisen, H.W., (In: Microbiology, 3rd Ed., Harper & Row, Philadelphia, PA (1980), pp. 290-295 and 381-418). Leukocytes must be able to attach to endothelial cells so that they can migrate from circulation to sites of ongoing inflammation. Furthermore, they must attach to antigen-presenting cells so that a normal specific immune response can occur, and finally, they must attach to appropriate target cells so that lysis of virally-infected or tumor cells can occur.

15

Leukocyte surface molecules involved in mediating such attachments have been identified using hybridoma technology. Briefly, monoclonal antibodies ("MAbs") directed against human T-cells (Davignon, D. et al., Proc. Natl. Acad. Sci. USA 78:4535-4539 (1981)) and mouse spleen cells (Springer, T. et al., Eur. J. Immunol. 9:301-306 (1979)) were identified which bound to leukocyte surfaces and inhibited the attachment related functions described above (Springer, T. et al., Fed. Proc. 44:2660-2663 (1985)). The molecules identified by those antibodies were called Mac-1 and Lymphocyte Function-associated Antigen-1 (LFA-1). Mac-1 is found on macrophages, granulocytes and large granular lymphocytes. LFA-1 is found on most lymphocytes (Springer, T.A., et al., Immunol. Rev. 68:111-135 (1982)). These two molecules, plus a third molecule, p150,95 (which has a tissue distribution similar to Mac-1) play a role in cellular adhesion (Keizer, G. et al., Eur. J. Immunol. 15:1142-1147 (1985)). Molecules such as those of LFA-1 family, which are involved in the process of cellular adhesion are referred to as "adhesion molecules."

25

The above-described leukocyte molecules were found to be structurally similar to one another, and to constitute members of a related family of glycoproteins (Sanchez-Madrid, F. et al., J. Exper. Med. 158:1785-1803 (1983); Keizer, G.D. et al., Eur. J. Immunol. 15:1142-1147 (1985)). This glycoprotein family is composed of heterodimers having one alpha subunit and one beta subunit. Although the alpha subunit of each of the antigens differed from one another, the beta subunit was found to be highly conserved (Sanchez-Madrid, F. et al., J. Exper. Med. 158:1785-1803 (1983)). The beta subunit of the glycoprotein family (referred to as "CD18" family) was found to have a molecular weight of 95 kd whereas the alpha subunits were found to vary from 150 kd to 180 kd (Springer, T., Fed. Proc. 44:2660-2663 (1985)). Although the alpha subunits of the membrane proteins do not share the extensive homology shared by the beta subunits, close analysis of the alpha subunits of the glycoproteins has revealed that there are substantial similarities between them. Reviews of the similarities between the alpha and beta subunits of the LFA-1 related glycoproteins are provided by Sanchez-Madrid, F. et al., J. Exper. Med. 158:586-602 (1983); J. Exper. Med. 158:1785-1803 (1983)).

30

A group of individuals has been identified who are unable to express normal amounts of any member of this adhesion protein family on their leukocyte cell surface (Anderson, D.C., et al., Fed. Proc. 44:2671-2677 (1985); Anderson, D.C., et al., J. Infect. Dis. 152:668-689 (1985)). The condition is known as "Leukocyte Adhesion Deficiency" or "LAD" syndrome. Leukocytes from these patients displayed *in vitro* defects similar to normal counterparts whose CD18 family of molecules had been antagonized by antibodies. Furthermore, these individuals are unable to mount a normal immune response due to an inability of their cells to adhere to cellular substrates (Anderson, D.C., et al., Fed. Proc. 44:2671-2677 (1985); Anderson, D.C., et al., J. Infect. Dis. 152:668-689 (1985)). LAD individuals present clinically with delayed umbilical cord separation, recurring and progressive soft tissue infections, and impaired pus formation, despite a striking blood leukocytosis. Studies of LAD individuals have revealed that immune reactions are mitigated when lymphocytes are unable to adhere in a normal fashion due to the lack of functional adhesion molecules of the CD18 family.

35

Thus, in summary, the ability of leukocytes, especially lymphocytes to maintain the health and viability of an animal requires that they be capable of adhering to other cells (such as endothelial cells). This adherence has been found to require cell-cell contacts which involve specific receptor molecules present on the cell surface of the lymphocytes. These receptors enable a lymphocyte to adhere to other lymphocytes or to endothelial, and other non-vascular cells. The cell surface receptor molecules have been found to be highly related to one another. Humans whose lymphocytes lack these cell surface receptor molecules exhibit defective antibody responses, chronic and recurring infections, as well as other clinical symptoms.

B. Asthma: Clinical Characteristics

Asthma is a heterogeneous family of diseases. It is characterized by a hyper-responsiveness of the tracheobronchi to stimuli (McFadden, E.R. et al., In: *Harrison's Principles of Internal Medicine*, 10th Ed., Petersdorf, R.G. et al., Eds., McGraw-Hill, NY (1983), pages 1512-1519); Kay, A.B., *Allergy and Inflammation*, Academic Press, NY (1987); which references are incorporated herein by reference). Clinically, asthma is manifested by the extensive narrowing of the tracheobronchi, by thick tenacious secretions, by paroxysms of dyspnea, cough, and wheezing. Although the relative contribution of each of these conditions is unknown, the net result is an increase in airway resistance, hyperinflation of the lungs and thorax, abnormal distribution of ventilation and pulmonary blood flow. The disease is manifested in episodic periods of acute symptoms interspersed between symptom-free periods. The acute episodes result in hypoxia, and can be fatal. Approximately 3% of the general world population suffers from the disease.

Two types of asthma have been described: allergic asthma and idiosyncratic asthma. Allergic asthma is usually associated with a heritable allergic disease, such as rhinitis, urticaria, eczema, etc. The condition is characterized by positive wheal-and-flare reactions to intradermal injections of airborne antigens (such as pollen, environmental or occupational pollutants, etc.), and increased serum levels of IgE. The development of allergic asthma appears to be causally related to the presence of IgE antibodies in many patients. Asthma patients who do not exhibit the above-described characteristics are considered to have idiosyncratic asthma.

Allergic asthma is believed to be dependent upon an IgE response controlled by T and B lymphocytes and activated by the interaction of airborne antigen with mast cell-bound pre-formed IgE molecules. The antigenic encounter must have occurred at concentrations sufficient to lead to IgE production for a prolonged period of time in order to sensitize an individual. Once sensitized, an asthma patient may exhibit symptoms in response to extremely low levels of antigen.

Asthma symptoms may be exacerbated by the presence and level of the triggering antigen, environmental factors, occupational factors, physical exertion, and emotional stress.

Asthma may be treated with methylxanthines (such as theophylline), beta-adrenergic agonists (such as catecholamines, resorcinols, saligenins, and ephedrine), glucocorticoids (such as hydrocortisone), inhibitors of mast cell degranulation (i.e. chromones such as cromolyn sodium) and anticholinergics (such as atropine).

C. Asthma: Immunological Characteristics

Asthma is believed to involve an influx of eosinophils ("eosinophilia") into the tissues of the lung (Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527-537 (1986), which reference is incorporated herein by reference). Eosinophils contain an arginine-rich, strongly basic protein which has been termed "MBP" (Gleich, G.J. et al., *J. Exper. Med.* 137:1459 (1973)). MBP comprises more than 50% of the granule protein of the eosinophil.

MBP was found to be toxic to normal mammalian cells, and to be expressed at toxic levels in tissues evidencing eosinophilia, such as the lung tissue of asthma patients (Gleich, G.J. et al., *J. Immunol.* 123:2925 (1975)). The linkage between MBP expression and asthma was strengthened by the discovery that the MBP levels found in the sputum of asthma patients was elevated relative to normal individuals (Frigas, E. et al., *Mayo Clin. Proc.* 56:345 (1981)). Since the discovery of MBP, other cytotoxic eosinophil proteins have been identified (Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527-537 (1986)).

Insight into the immunological basis of asthma has been gained from the above-described studies, from bronchoalveolar lavage studies (Godard, P. et al., *J. Allergy Clin. Immunol.* 70:88 (1982)), and studies of respiratory smooth muscle denuded of epithelium (Flavahan, N.A. et al., *J. Appl. Physiol.* 58:834 (1985); Barnes, P. J. et al., *Br. J. Pharmacol.* 86:685 (1985)). Although these studies have not led to the elucidation of the mechanism underlying the immunology of asthma, they have led to the development of a generally accepted hypothesis concerning the immunological etiology of the disease (see, Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527-537 (1986)).

The hallmarks of the pathology of asthma are a massive infiltration of the lung parenchyma by eosinophils and the destruction of mucociliary capacity. The "eosinophil hypothesis" suggests that eosinophils are attracted to the bronchus in order to neutralize harmful mediators released by the mast cells of the lung. According to the hypothesis eosinophils are attracted to the bronchi where they degranulate to release MBP and other cytotoxic molecules. Upon degranulation, eosinophils release enzymes such as histaminase, arylsulfatase and phospholipase D which enzymatically neutralize the harmful mediators of the mast cell. These molecules also promote the destruction of the mucociliary apparatus, and thus prevent the clearing of the bronchial secretions, and contribute to the lung damage characteristic of asthma.

Thus, it is believed that asthma is caused by the eosinophils which invade the bronchi in an abnormal response to primary effects of antigen presence. The MBP of the eosinophils damage the epithelial cells of the

bronchi. Leukotrienes and Platelet Activating Factor ("PAF") are produced by the eosinophil, and result in bronchial dilation. Molecules (such as MBP) released by the eosinophil can also activate mast cells to release leukotrienes and histamine, thereby causing both bronchospasm and increased eosinophilia.

In view of the clinical importance of asthma, it is highly desirable to identify new or improved therapies for 5 treating asthma patients.

The present invention relates to the use of intercellular adhesion molecules ("ICAMs"), such as ICAM-1, and the functional derivatives of such ICAMs in the treatment of asthma. The invention further concerns the use of molecules (such as antibodies, or antibody fragments, or receptor molecules) that are able to bind to an intercellular adhesion molecule (or a derivative of an ICAM molecule) in the treatment of asthma. The invention 10 also includes an assay for detecting agents having therapeutic potential in the treatment of asthma.

The invention additionally pertains to a method for treating asthma in a patient which comprises providing to the patient an effective therapeutic amount of an agent selected from the group consisting of:

- (a) an antibody capable of binding to ICAM-1;
- (b) a fragment of the antibody (a), the fragment being capable of binding to ICAM-1;
- (c) ICAM-1, being substantially free of natural contaminants;
- (d) a functional derivative of ICAM-1;
- (e) an antibody capable of binding to a member of the CD18 family of glycoproteins;
- (f) a fragment of the antibody (e), the fragment being capable of binding to a member of the CD18 family of glycoproteins;
- (g) a member of the CD18 family of glycoproteins, being substantially free of natural contaminants; and
- (h) a functional derivative of a member of the CD18 family of glycoproteins.

The invention further provides a method of identifying an agent capable of therapeutic potential in the treatment of asthma which comprises administering the agent to a non-human mammal (especially a primate) having received multiple inhalations of an antigen and then measuring any increase in airway responsiveness.

25 Figure 1 shows the nucleotide and amino acid sequence of ICAM-1 cDNA. The first ATG is at position 58. Translated sequences corresponding to ICAM-1 tryptic peptides are underlined. The hydrophobic putative signal peptide and transmembrane sequences have a bold underline. N-linked glycosylation sites are boxed. The polyadenylation signal AATAAA at position 2976 is over-lined. The sequence shown is for the HL-60 cDNA clone. The endothelial cell cDNA was sequenced over most of its length and showed only minor differences.

30 Figure 2 shows the domain structure of ICAM-1.
Figure 3 shows eosinophil adherence to flat bottom tissue culture plate wells protein-coated with no stimulus, protein-coated with PAF (10^{-7} M) stimulus, immune complex (IC) coated and stimulus, and non-coated with no stimulus. Adhered cells were quantitated by a colorimetric assay for eosinophil peroxidase (EPO) as mean EPO units \pm S.D.

35 Figure 4 shows the effect of various monoclonal antibodies (MAbs) (supernatant 1:4 dilution) on eosinophil adherence to flat bottom tissue culture plate wells coated with immune complexes (IC). Adhered cells were quantitated by a colorimetric assay for eosinophil peroxidase (mean EPO units \pm S.D.). Statistically significant attenuation of adherence is signified by an asterisk.

40 Figure 5 shows the effect of various monoclonal antibodies (MAbs) (supernatant 1:4 dilution) on PAF (10^{-7} M) induced eosinophil adherence to LPS (10 ng/ml)-stimulated and glutaraldehyde-fixed endothelium. Adhered cells were quantitated by a colorimetric assay for eosinophil peroxidase (EPO units \pm S.D.). Statistically significant attenuation of adherence is signified by an asterisk.

45 Figure 6 shows the effects of anti-ICAM-1 (RR1/1), anti-LFA-1 beta (R15.7) and anti-HLA class 1 (W6/32) monoclonal antibodies (supernatant 1:4 dilution) on (A) platelet-activating factor (PAF, 10^{-7} M) induced eosinophil adhesion to lipopolysaccharide (LPS, 10 ng/ml)-stimulated human umbilical vein endothelium, and (B) eosinophil adhesion to flat bottom tissue culture plate wells coated with Ascaris extract (no stimulus) or immune complex (IC stimulus). Statistically significant attenuation of adherence is signified by an asterisk.

50 Figure 7 shows the effect of the anti-ICAM-1 monoclonal antibody R6.5 on the (A) airway eosinophil infiltration and (B) increase in airway responsiveness (decrease in methacholine PC₁₀₀) induced by three alternate day inhalations of Ascaris in Ascaris-sensitive cynomolgus monkeys (Macaca fascicularis). Studies with R6.5 treatment are compared to bracketing control studies performed on each animal.

55 Figure 8 shows changes induced by a single inhalation of antigen in (A) inhaled methacholine PC₁₀₀, (B) bronchoalveolar lavage (BAL) eosinophils, (C) BAL neutrophils, (D) BAL macrophages/monocytes, and (E) BAL lymphocytes. Xsq = Kruskal-Wallis Test (Chi-square approximation). NSD = no significant difference. Each letter represents an individual monkey.

Figure 9 shows changes induced by multiple inhalations of antigen in (A) inhaled methacholine PC₁₀₀ and

(B) bronchoalveolar lavage (BAL) eosinophils. X_{sq} = Kruskal-Wallis Test (Chi-square approximation). Each letter represents an individual monkey.

A. The Immunopathology of Asthma

5 As indicated above, one of the most important and characteristic features of asthma is the extreme (10 to 1000 times normal) sensitivity of the bronchi to inhaled agents (physical, chemical and physiological) (Boushey, H.A. et al., *Am. Rev. Respir. Dis.* 121:389 (1980); Hargreave, F.E. et al., *J. Allergy Clin. Immunol.* 68:347 (1981)). The severity of this "airway hyperresponsiveness" (which is measured clinically by determining the responsiveness of the airways to inhaled histamine, methacholine, cold air or to exercise) correlates with the intensity of asthmatic symptoms (Hargreave, F.E. et al., *J. Allergy Clin. Immunol.* 68:347 (1981); Boulet, L-P et al., *J. Allergy Clin. Immunol.* 71:399 (1983); Chan-Yeung, M. et al., *Am. J. Med.* 72:411 (1982)), diurnal variations in peak flow rates (Ryan G. et al., *Thorax* 37:423 (1982)) and therapy required (Hargreave, F.E. et al., *J. Allergy Clin. Immunol.* 68:347 (1981); Juniper, E.F. et al., *Thorax* 36:575 (1981)).

15 Although airway hyperresponsiveness in asthmatics can remain stable over several years (Juniper E.F. et al., *Thorax* 37:288-291 (1982)), responsiveness has been shown to be increased by exposure to allergens (Boulet L.-P. et al., *J. Allergy Clin. Immunol.* 71:399-406 (1983), Cartier A. et al., *J. Allergy Clin. Immunol.* 70:170-177 (1982), Cockcroft D.W. et al., *Clinical Allergy* 7:503-513 (1977), Gundel R.H. et al., *Amer. Rev. Respir. Dis.* (1989), Lanes S. et al., *J. Appl. Physiol.* 61:864-872 (1986), Marsh W.R. et al., *Amer. Rev. Respir. Dis.* 131:875-879 (1985), Sotomayor H. et al., *Amer. Rev. Respir. Dis.* 130:56-58 (1984)), air pollutants (Golden J.A. et al., *Amer. Rev. Respir. Dis.* 118:287-294 (1978)), viral infections (Empey D.W. et al., *Amer. Rev. Respir. Dis.* 113:131-139 (1976)) and certain occupational chemicals (Chan-Yeung M. et al., *Amer. J. Med.* 72:411-415 (1982), Durham S.R. et al., *J. Allergy Clin. Immunol.* 79:398-406 (1987), Lam S. et al., *J. Allergy Clin. Immunol.* 72:134-139 (1983), Lam S. et al., *J. Allergy Clin. Immunol.* 63:28-34 (1979)). In fact, there is evidence to suggest that airway hyperresponsiveness is a consequence rather than a predisposing factor of asthma (Boulet L.-P. et al., *J. Allergy Clin. Immunol.* 71:399-406 (1983), Chan-Yeung M. et al., *Amer. J. Med.* 72:411-415 (1982), Gundel R.H. et al., *Amer. Rev. Respir. Dis.* (1989), Marsh W.R. et al., *Amer. Rev. Respir. Dis.* 131:875-879 (1985), Empey D.W. et al., *Amer. Rev. Respir. Dis.* 113:131-139 (1976), Lam S. et al., *J. Allergy Clin. Immunol.* 63:28-34 (1979)).

20 30 While the mechanisms underlying the pathogenesis of airway hyperresponsiveness are not known, results from many studies suggest, as indicated above, that eosinophil infiltration and desquamation of the bronchial epithelium are involved (DeMonchy, J.G.R. et al., *Am. Rev. Respir. Dis.* 131:373 (1985); Laitinen, L.A. et al., *Am. Rev. Respir. Dis.* 137:62 (1988)). Since eosinophil mediators have been shown to damage airway epithelial cells *in vitro* these two events may be linked (Frigas, E. et al., *J. Allergy Clin. Immunol.* 77:527 (1986)).

B. Asthma and Intercellular Adhesion

35 The present invention derives, in part, from the development of an assay capable of identifying agents having therapeutic potential in the treatment of asthma.

40 As used herein, "asthma" refers to either allergic or idiosyncratic asthma. An agent is said to have a therapeutic potential in the treatment of asthma if it may lessen (i.e. attenuate) the severity, extent or duration of the asthma symptoms. Such agents are preferably identified through the use of the following "asthma model system". As used herein, an agent is said to be able to treat asthma if, when administered to a patient, the agent is capable of attenuating either the severity, extent or duration of the asthma symptoms.

45 One aspect of the present invention derives from the discovery that the migration of eosinophils into the lung is dependent upon intercellular adhesion, and specifically that such adhesion is dependent upon an "ICAM-1" ("Intercellular Adhesion Molecule-1") interaction.

50 As used herein, a molecule is a member of the CD18 family of glycoproteins if it contains either an alpha subunit of a member of the CD18 family of glycoproteins (i.e. a CD11 subunit), a beta subunit of a member of the CD18 family of glycoproteins (i.e. a CD18 beta subunit), or both an alpha and a beta subunit of a member of the CD18 family of glycoproteins. Thus, as used herein, a member of the CD18 family of glycoproteins includes molecules having only one subunit of a CD18 member as well as heterodimer (i.e. a molecule having both an alpha and a beta subunit of a member of the CD18 family. All such molecules may be either bound to a membrane or solid support or unbound (i.e. "soluble").

55 "ICAM-1" is the natural ligand for the CD18 family of glycoprotein receptor molecules (Rothlein, R. et al., *J. Immunol.* 137:1270 (1986); Marlin, S.D. et al., *Cell* 51:813 (1987)). ICAM-1 is a 76-97 Kd glycoprotein. ICAM-1 is not a heterodimer. The identification, characterization, and amino acid sequence of ICAM-1, and the production of antibody reactive with ICAM-1 and other adhesion molecules are disclosed in European Patent

Application Serial No. 289,949 (which reference is incorporated herein by reference) and in Rothlein, R. *et al.* (*J. Immunol.* 137:1270-1274 (1986)), Smith, G.W. *et al.*, in *Structure and Function of Molecules Involved in Leukocyte Adhesion*, A.S. Rosenthal, *et al.*, *Eds.* (Springer-Verlag, New York, 1989); Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988) and Barton, R.W. *et al.*, *J. Immunol.* 143, (1989)), all of which references are incorporated herein by reference).

In brief, ICAM-1 is a cell surface glycoprotein expressed on non-hematopoietic cells such as vascular endothelial cells, thymic epithelial cells, certain other epithelial cells, and fibroblasts, and on hematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal centered B cells and dendritic cells in tonsils, lymph nodes, and Peyer's patches. ICAM-1 is highly expressed on vascular endothelial cells in T cell areas in lymph nodes and tonsils showing reactive hyperplasia. ICAM-1 is expressed in low amounts on peripheral blood lymphocytes. ICAM-1 appears to be required for neutrophil migration into inflamed tissues. Phorbol ester-stimulated differentiation of some myelomonocytic cell lines greatly increases ICAM-1 expression. Thus, ICAM-1 is preferentially expressed at sites of inflammation, and is not generally expressed by quiescent cells. ICAM-1 expression on dermal fibroblasts is increased threefold to fivefold by either interleukin 1 or gamma interferon at levels of 10 U/ml over a period of 4 or 10 hours, respectively. The induction is dependent on protein and mRNA synthesis and is reversible.

ICAM-1 displays molecular weight heterogeneity in different cell types with a molecular weight of 97 kd on fibroblasts, 114 kd on the myelomonocytic cell line U937, and 90 kd on the B lymphoblastoid cell JY. ICAM-1 biosynthesis has been found to involve an approximately 73 kd intracellular precursor. The non-N-glycosylated form resulting from tunicamycin treatment (which inhibits glycosylation) has a molecular weight of 55 kd.

ICAM-1 isolated from phorbol ester stimulated U937 cells or from fibroblast cells yields an identical major product having a molecular weight of 60 kd after chemical deglycosylation. ICAM-1 monoclonal antibodies interfere with the adhesion of phytohemagglutinin blasts to LFA-1 deficient cell lines. Pretreatment of fibroblasts, but not lymphocytes, with monoclonal antibodies capable of binding ICAM-1 inhibits lymphocyte-fibroblast adhesion. Pretreatment of lymphocytes, but not fibroblasts, with antibodies against LFA-1 has also been found to inhibit lymphocyte-fibroblast adhesion.

ICAM-1 is, thus, the binding ligand of molecules of the CD18 family of glycoproteins. It is inducible on fibroblasts and endothelial cells *in vitro* by inflammatory mediators such as IL-1, gamma interferon and tumor necrosis factor in a time frame consistent with the infiltration of lymphocytes into inflammatory lesions *in vivo* (Dustin, M.L., *et al.*, *J. Immunol.* 137:245-254, (1986); Prober, J.S., *et al.*, *J. Immunol.* 137:1893-1896, (1986)). Further ICAM-1 is expressed on non-hematopoietic cells such as vascular endothelial cells, thymic epithelial cells, other epithelial cells, and fibroblasts and on hematopoietic cells such as tissue macrophages, mitogen-stimulated T lymphocyte blasts, and germinal center B-cells and dendritic cells in tonsils, lymph nodes and Peyer's patches (Dustin, M.L., *et al.*, *J. Immunol.* 137:245-254, (1986)). ICAM-1 is expressed on keratinocytes in benign inflammatory lesions such as allergic eczema, lichen planus, exanthema, urticaria and bullous diseases. Allergic skin reactions provoked by the application of a hapten on the skin to which the patient is allergic also revealed a heavy ICAM-1 expression on the keratinocytes. On the other hand toxic patches on the skin did not reveal ICAM-1 expression on the keratinocytes. ICAM-1 is present on keratinocytes from biopsies of skin lesions from various dermatological disorders and ICAM-1 expression is induced on lesions from allergic patch tests while keratinocytes from toxic patch test lesions failed to express ICAM-1.

Yet another aspect of the invention is the discovery that agents which prevent or inhibit cellular adhesion may be employed in the treatment of asthma.

One example of agents which may be used in accordance with the present invention are ICAM-1 and functional derivatives of ICAM-1. Since ICAM-1 mediates cellular adhesion by binding to a receptor molecule on the eosinophil cell surface, functional derivatives of ICAM-1 which can bind to the ICAM-1 receptor present on eosinophils will compete with the ICAM-1 on lung endothelial cells, thus attenuating cellular adhesion of the eosinophils, and providing a treatment for asthma.

A "functional derivative" of ICAM-1 is a compound which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of ICAM-1. The term "functional derivatives" is intended to include the "fragments," "variants," "analogs," or "chemical derivatives" of a molecule. A "fragment" of a molecule such as ICAM-1, is meant to refer to any polypeptide subset of the molecule. Fragments of ICAM-1 which have ICAM-1 activity and which are soluble (i.e. not membrane bound) are especially preferred.

ICAM-1 is composed of 7 domains (Staunton, D.E. *et al.*, *Immunol. Today* 9:213-215 (1988); Staunton, D.E. *et al.*, *Cell* 52:925-934 (1988); Staunton, D.E. *et al.*, *Cell* 56:849-854 (1989); Staunton, D.E. *et al.*, *Tissue Antigens* 33:287 (1989), all of which references are incorporated herein by reference). The domains of ICAM-1 are shown in Figure 2. Domains 1 and 2 have been found to be important for the binding of ICAM-1 to its receptor molecule (Staunton, D.E. *et al.*, *Tissue Antigens* 33:286 (1989); Staunton, D.E. *et al.*, *FASEB J.* 3:A446 (1989) both of which references are incorporated herein by reference). In accordance with the present invention, ICAM-

1 functional derivatives, and especially such derivatives which comprise fragments or mutant variants of ICAM-1 which possess both domains 1 and 2 can be used in the treatment or therapy of asthma. More preferred for such treatment or therapy are ICAM-1 fragments or mutant variants which contain domain 2 of ICAM-1. Most preferred for such treatment or therapy are ICAM-1 fragments or mutant variants which contain domain 1 of ICAM-1.

5 A "variant" of a molecule such as ICAM-1 is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof.

10 A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules not found in the other, or if the sequence of amino acid residues is not identical. An "analog" of a molecule such as ICAM-1 is meant to refer to a molecule substantially similar in function to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may 15 improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). "Toxin-derivatized" molecules constitute a special class of "chemical derivatives." A "toxin-derivatized" molecule is a 20 molecule (such as ICAM-1 or an antibody) which contains a toxin moiety. The binding of such a molecule to a cell brings the toxin moiety into close proximity with the cell and thereby promotes cell death. Any suitable toxin moiety may be employed; however, it is preferable to employ toxins such as, for example, the ricin toxin, the diphtheria toxin, radioisotopic toxins, membrane-channel-forming toxins, etc. Procedures for coupling such moieties to a molecule are well known in the art.

25 An additional example of agents which may be used in accordance with the present invention to treat asthma are LFA-1, Mac-1 or p150,95, or the functional derivatives of these molecules. Such molecules and their functional derivatives can provide a treatment for asthma by virtue of their capacity to bind to the ICAM-1 of endothelial cells, and thus impair the ability of such cells to mediate binding and adhesion of eosinophils.

30 Of special interest to the present invention are functional derivatives of LFA-1, Mac-1 or p150,95 which are soluble molecules. Of special interest are functional derivatives of these molecules which are heterodimers (containing both the alpha and beta subunits of the molecules) and monomeric derivatives capable of binding ICAM-1. Soluble heterodimers are especially preferred.

35 ICAM-1 and the members of the CD18 family of molecules are immunogenic molecules. Thus, it is possible to obtain antibodies capable of binding to ICAM-1 or members of the CD18 family of molecules. Such antibodies may be used in accordance with the methods of the present invention in the treatment of asthma.

40 Such antibodies may be obtained by introducing either the purified molecules (or cells which naturally express these molecules) into an appropriate animal, as by intraperitoneal injection, etc. If desired, the serum of such an animal may be removed and used as a source of polyclonal antibodies capable of binding these molecules. It is, however, preferable to remove splenocytes from such animals, to fuse such spleen cells with a myeloma cell line and to permit such fusion cells to form a hybridoma cell which secretes monoclonal antibodies capable of binding ICAM-1 or members of the CD18 family of molecules.

45 The hybridoma cells, obtained in the manner described above may be screened as described above to identify desired hybridoma cells that secrete antibody capable of binding either to ICAM-1 or to members of the CD18 family of molecules (either the alpha or beta subunit).

50 Since such antibodies have the capacity to bind to ICAM-1 or its receptor, they (and their fragments having antigen binding ability, such as Fab, F(ab)₂, etc.) may be used to attenuate cellular adhesion, and thus provide an additional example of an agent which may be used in accordance with the present invention to treat asthma.

55 As indicated above, both polyclonal and monoclonal antibodies may be employed in accordance with the present invention. Of special interest to the present invention are antibodies to ICAM-1 (or their functional derivatives), or to members of the CD18 family (or their functional derivatives), which are produced in humans, or are "Humanized" (i.e. non-immunogenic in a human) by recombinant or other technology. Humanized antibodies may be produced, for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (i.e. chimeric antibodies) (Robinson, R.R. *et al.*, International Patent Application PCT/US86/02269; Akira, K. *et al.*, European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison, S.L. *et al.*, European Patent Application 173,494; Neuberger, M.S. *et al.*, European Patent Application 125,023; Better, M. *et al.*, *Science* PCT Application WO 86/01533; Cabilly, S. *et al.*, European Patent Application 125,023; Better, M. *et al.*, *Science* 240:1041-1043 (1988); Liu, A.Y. *et al.*, *Proc. Natl. Acad. Sci. USA* 84:3439-3443 (1987); Liu, A.Y. *et al.*, *J. Immunol.* 139:3521-3526 (1987); Sun, L.K. *et al.*, *Proc. Natl. Acad. Sci. USA* 84:214-218 (1987); Nishimura, Y. *et al.*, *Canc. Res.* 47:999-1005 (1987); Wood, C.R. *et al.*, *Nature* 314:446-449 (1985); Shaw *et al.*, *J.*

Natl. Cancer Inst. 80:1553-1559 (1988).

General reviews of "humanized" chimeric antibodies are provided by Morrison, S.L. (Science, 229:1202-1207 (1985)) and by Oi, V.T. et al., *BioTechniques* 4:214 (1986)).

Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (Jones, P.T. et al., *Nature* 321:552-525 (1986); Verhoeyan et al., *Science* 239:1534 (1988); Beidler, C.B. et al., *J Immunol.* 141:4053-4060 (1988)).

The anti-asthma agents of the present invention may be obtained by natural processes (such as, for example, by inducing an animal, plant, fungi, bacteria, etc., to produce a non-immunoglobulin antagonist of ICAM-1, or by inducing an animal to produce polyclonal antibodies capable of binding to ICAM-1); by synthetic methods (such as, for example, by using the Merrifield method for synthesizing polypeptides to synthesize ICAM-1, functional derivatives of ICAM-1, or protein antagonists of ICAM-1 (either immunoglobulin or non-immunoglobulin)); by hybridoma technology (such as, for example, to produce monoclonal antibodies capable of binding to ICAM-1); or by recombinant technology (such as, for example, to produce the anti-asthma agents of the present invention in diverse hosts (i.e., yeast, bacteria, fungi, cultured mammalian cells, etc.), or from recombinant plasmids or viral vectors), or by proteolysis. The choice of which method to employ will depend upon factors such as convenience, desired yield, etc. It is not necessary to employ only one of the above-described methods, processes, or technologies to produce a particular anti-asthma agent; the above-described processes, methods, and technologies may be combined in order to obtain a particular anti-asthma agent.

Functional derivatives of ICAM-1, or a member of the CD18 family, having up to about 100 residues may be conveniently prepared by in vitro synthesis. If desired, such fragments may be modified by reacting targeted amino acid residues of the purified or crude protein with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The resulting covalent derivatives may be used to identify residues important for biological activity. In the embodiments listed below, this aspect of the invention is described with reference to the functional derivatives of ICAM-1. Such methods may also be employed to produce functional derivatives of any member of the CD18 family of molecules.

Cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful, the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues *per se* has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($\text{R}'-\text{N}-\text{C}-\text{N}-\text{R}''$) such as 1-cydoethyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3 (4 azonia 4,4-dimethylpentalyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking an ICAM-1 functional derivative molecule to a water-insoluble support matrix or surface for use in the method for cleaving an ICAM-1 functional derivatives fusion polypeptide to release and recover the cleaved polypeptide. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivati-

zing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

5 Glutamyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

10 Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecule Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

15 Functional derivatives of ICAM-1 having altered amino acid sequences can also be prepared by mutations in the DNA. The nucleotide sequence which encodes the ICAM-1 gene is shown in Figure 1. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown in Figure 1. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see EP Patent Application Publication No. 75,444).

20 At the genetic level, these functional derivatives ordinarily are prepared by site-directed mutagenesis of nucleotides in the DNA encoding the ICAM-1 molecule, thereby producing DNA encoding the functional derivative, and thereafter expressing the DNA in recombinant cell culture. The functional derivatives typically exhibit 25 the same qualitative biological activity as the naturally occurring analog. They may, however, differ substantially in such characteristics with respect to the normally produced ICAM-1 molecule.

25 While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed ICAM-1 functional derivatives 30 screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, site-specific mutagenesis.

35 Preparation of an ICAM-1 functional derivative molecule in accordance herewith is preferably achieved by site-specific mutagenesis of DNA that encodes an earlier prepared functional derivatives or a nonvariant version of the protein. Site-specific mutagenesis allows the production of ICAM-1 functional derivatives through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well 40 as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman *et al.*, DNA 2:183 (1983), the disclosure of which is incorporated herein by reference.

45 As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, Third Cleveland Symposium on Macromolecules and Recombinant DNA, Editor A. Walton, Elsevier, Amsterdam (1981), the disclosure of which is incorporated herein by reference. These phage are readily commercially available and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin 50 of replication (Veira *et al.*, Meth. Enzymol. 153:3 (1987)) may be employed to obtain single-stranded DNA.

55 In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al.*, Proc. Natl. Acad. Sci. (USA) 75:5765 (1978). This primer is then annealed with the single-stranded protein-sequence-containing vector, and subjected to DNA-polymerizing enzymes such as E. coli polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as JM101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated protein region may be removed and placed in an appropriate

vector for protein production, generally an expression vector of the type that may be employed for transformation of an appropriate host.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably 1 to 10 residues, and typically are contiguous.

5 Amino acid sequence insertions include amino and/or carboxyl-terminal fusions of from one residue to polypeptides of essentially unrestricted length, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the complete ICAM-1 molecule sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5. An example of a terminal insertion includes a fusion of a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus of the molecule to facilitate the secretion of the ICAM-1 functional derivative from recombinant hosts.

10 The third group of functional derivatives are those in which at least one amino acid residue in the ICAM-1 molecule, and preferably, only one, has been removed and a different residue inserted in its place. Such substitutions preferably are made in accordance with the following Table 1 when it is desired to modulate finely the characteristics of the ICAM-1 molecule.

15

TABLE 1

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
20	Ala	gly; ser
	Arg	lys
	Asn	gln; his
	Asp	glu
25	Cys	ser
	Gln	asn
	Glu	asp
	Gly	ala; pro
	His	asn; gln
30	Ile	leu; val
	Leu	ile; val
	Lys	arg; gln; glu
	Met	leu; tyr; ile
	Phe	met; leu; tyr
35	Ser	thr
	Thr	ser
	Trp	tyr
	Tyr	trp; phe
	Val	ile; leu

40 Substantial changes in functional or immunological identity are made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the 45 side chain. The substitutions that in general are expected to those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, e.g., lysyl, 50 arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, e.g., glutamyl or aspartyl; or (e) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine.

Most deletions and insertions, and substitutions in particular, are not expected to produce radical changes in the characteristics of the ICAM-1 molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a functional derivative typically is made by site-specific mutagenesis of the native ICAM-1 molecule-encoding nucleic acid, expression of the variant nucleic acid in recombinant cell culture, and, optionally, purification from the cell culture, for example, by immunoaffinity adsorption on an anti-ICAM-1 molecule antibody column (to absorb the functional derivative by binding it to at

least one remaining immune epitope).

The activity of the cell lysate or purified ICAM-1 molecule functional derivative is then screened in a suitable screening assay for the desired characteristic. For example, a change in the immunological character of the functional derivative, such as affinity for a given antibody, is measured by a competitive type immunoassay.

5 Changes in immunomodulation activity are measured by the appropriate assay. Modifications of such protein properties as redox or thermal stability, biological half-life, hydrophobicity, susceptibility to proteolytic degradation or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

10 **C. Administration of the Compositions of the Present Invention**

The therapeutic effects of the anti-asthma agents of the present invention may be obtained by providing such agents to a patient by any suitable means (i.e. intravenously, intramuscularly, subcutaneously, enterally, or parenterally). It is preferred to administer the agents of the present invention intranasally as by nasal spray, 15 swab, etc. It is especially preferred to administer such agents by oral inhalation, or via an oral spray or oral aerosol. When administering agents by injection, the administration may be by continuous infusion, or by single or multiple boluses.

20 The therapeutic advantages of any of the above-discussed agents can be augmented through the use of functional derivatives possessing additional amino acid residues added to enhance coupling to carrier or to enhance the activity of the agent. The scope of the present invention is further intended to include functional derivatives of ICAM-1 which lack certain amino acid residues, or which contain altered amino acid residues, so long as such derivatives exhibit the capacity to affect cellular adhesion.

25 The antibodies of the present invention and the ICAM-1 molecule and the members of the CD18 family disclosed herein are said to be "substantially free of natural contaminants" if preparations which contain them are substantially free of materials with which these products are normally and naturally found.

The present invention extends to the use of antibodies, and biologically active fragments thereof, (whether polyclonal or monoclonal) which are capable of binding to ICAM-1 or to a member of the CD18 family in the treatment of asthma.

30 In providing a patient with antibodies, or fragments thereof, capable of binding to ICAM-1 or to a member of the CD18 family, or when providing ICAM-1 or a member of the CD18 family (or a fragment, variant, or derivative thereof) to a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered. The therapeutically 35 effective dose can be lowered by using combinations of the above-described agents (such as, for example, if anti-ICAM-1 antibody is additionally administered with an anti-LFA-1 antibody). As used herein, one compound is said to be additionally administered with a second compound when the administration of the two compounds is in such proximity of time that both compounds can be detected at the same time in the patient's serum.

40 The anti-asthma agents of the present invention are intended to be provided to recipient subjects in an amount sufficient to lessen or attenuate the severity, extent or duration of the asthma symptoms.

45 The antibody agents of the invention, or their fragments, may be administered either alone or in combination with one or more additional anti-asthma agents (such as methylxanthines (such as theophylline), beta-adrenergic agonists (such as catecholamines, resorcinols, saligenins, and ephedrine), glucocorticoids (such as hydrocortisone), chromones (such as cromolyn sodium) and anticholinergics (such as atropine), in order to decrease the amount of such agents needed to treat the asthma symptoms.

50 The administration of the agent(s) of the invention may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the agent(s) are provided in advance of any asthma symptom. The prophylactic administration of the agent(s) serves to prevent or attenuate any subsequent asthmatic response. When provided therapeutically, the agent(s) are provided at (or shortly after) the onset of a symptom of asthma. The therapeutic administration of the compound(s) serves to attenuate any actual asthmatic episode. The agents of the present invention may, thus, be provided either prior to the onset of an anticipated asthmatic episode (so as to attenuate the anticipated severity, duration or extent of the episode) or after the initiation of the episode.

55 A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The agents of the present invention can be formulated according to known methods to prepare phar-

5 maceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences (16th ed., Osol, A., Ed., Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of anti-ICAM antibody or ICAM-1 molecule, or their functional derivatives, together with a suitable amount of carrier vehicle.

10 Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb anti-ICAM-1 antibody or ICAM-1, or their functional derivatives. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine, sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate anti-ICAM-1 antibody or ICAM-1 molecules, or their functional derivatives, into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatine-microcapsules and poly(methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

15 Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

25

EXAMPLE 1

LUNG EOSINOPHIL ADHESION REACTIONS DEPEND ON CD11 AND ICAM-1 CELL ADHESION MOLECULES

30

In order to evaluate the requirement for cell adhesion of eosinophils in their migratory and cytotoxic function, eosinophil adhesion *in vitro* was tested. In particular, the role of the CD18 family of cellular adhesion molecules, and of ICAM-1, in primate eosinophil adhesion to protein coated plastic and to human endothelium was examined.

35

35 Eosinophils were obtained by bronchoalveolar lavage from adult male cynomolgus monkeys (*Macaca fascicularis*), purified (>93% by morphology) on a Percoll continuous density gradient, washed, and added to 96 well flat-bottom tissue culture plates at a concentration of 5×10^3 cells/well. After a 60 min. incubation at 37°C, the non-adherent cells were removed using a plate washer. Adherent cells were enumerated visually (no aggregation or degranulation was observed) and by a colorimetric assay which measures eosinophil peroxidase (EPO).

40

The eosinophils spontaneously adhered and spread on the bottom of untreated wells or of wells coated with immune complexes (made from *Ascaris* extract and serum from an *Ascaris*-immune monkey). In contrast, eosinophils did not adhere well to wells coated with proteins, including bovine serum albumin, normal monkey serum, or *Ascaris* extract. Of various soluble stimuli tested, Platelet Activating Factor (PAF) induced the most pronounced and consistent adherence of eosinophils to protein-coated wells (Figure 3).

45

The role of CD18 adhesion molecules and of ICAM-1 in this adherence process was tested using monoclonal antibodies reactive with each of these membrane glycoproteins. Antibodies used included: R3.3 and R15.7 (anti-CD18); R3.1 (anti-CD11a); M1/70 and LM2/1 (anti-CD11b); RR2/1 and R6.5.D6 (anti-ICAM-1); and W6/32 (anti HL-A class I).

50

Adhesion of the eosinophils to immune complex-coated wells appeared to be CD11b-dependent, since mAbs against CD11b and CD18 inhibited adhesion while mAbs against CD11a, ICAM-1, and HL-A did not (Figure 4). Identical results were obtained using eosinophils activated by soluble stimuli, including PAF, and protein-coated wells.

55

Adhesion of eosinophils to endothelial cells activated by LPS (10 ng/ml) and fixed with glutaraldehyde was also tested. Human umbilical vein endothelial cells were prepared as described by Smith, C.W. et al. (*J. Clin. Invest.* 82:1746 (1988) which reference is incorporated herein by reference). Adhesion of eosinophils induced by PAF (10^{-7} M) to such activated endothelial cells was partially inhibited by mAbs against CD11a, CD11b, and ICAM, completely inhibited by anti-CD18, and not inhibited by anti-HL-A (Figure 5). These experiments revealed

that the observed adhesion was partially dependent on CD11a, CD11b, and ICAM-1.

In summary, primate lung eosinophils behave nearly identically to human neutrophils in membrane adhesion reactions involving CD11a, CD11b, and ICAM-1. The CD18 family of CAMs therefore plays a major role in eosinophil adherence and is responsible to selective tissue accumulation of eosinophils in LAD patients.

5

EXAMPLE 2

ROLE OF ICAM-1 IN EOSINOPHIL INFILTRATION

10 In order to demonstrate that ICAM-1 contributed to the eosinophil infiltration, airway epithelium desquamation and increased airway responsiveness that characterize the airway inflammation underlying bronchial asthma, the effect of monoclonal antibodies reactive with ICAM-1 on asthma in primates was investigated. Specifically, (a) the contribution of ICAM-1 to eosinophil adhesion to vascular endothelium *in vitro*, (b) the induction of ICAM-1 on airway epithelium *in vitro* and *in vivo* as well as on bronchial vascular endothelium *in vivo*, 15 and (c) the contribution of ICAM-1 to the eosinophil infiltration and increase in airway responsiveness induced by multiple inhalations of antigen *in vivo* were investigated.

18 Primate lung eosinophils were stimulated with platelet-activating factor (PAF, 10⁻⁷M), and incubated in the presence of lipopolysaccharide (LPS, 10 ng/ml) stimulated cultured human umbilical vein endothelial cells (HUVECs) in order to assay for cellular adhesion. Eosinophils were obtained by bronchoalveolar lavage from 20 adult male cynomolgus monkeys (*Macaca fascicularis*) with airway eosinophilia, purified (morphologically >95% pure) on a Percoll continuous density gradient (Riding, G.A. *et al.*, *J. Immunol. Meth.* 46:113 (1981)), washed and added to 96 well flat bottom tissue culture plates (5 x 10³ cells/well). After a 60 minute incubation at 37°C, the non-adherent cells were removed by an automated plate washer. Adherent cells were quantitated visually and by a colorimetric assay for eosinophil peroxidase, EPO (Strath, M. *et al.*, *J. Immunol. Meth.* 83:209 25 (1985)). Human umbilical vein endothelial cells were isolated, cultured to a confluent monolayer in each well, stimulated for 4 hours with LPS and finally fixed in 1% glutaraldehyde (Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988)). Immune complex wells were made by coating with *Ascaris* extract followed by serum from an *Ascaris*-sensitive monkey.

28 Adhesion was found to be significantly inhibited by the anti-ICAM-1 monoclonal antibody RR1/1 (Rothlein, R. *et al.*, *J. Immunol.* 137:1270 (1986); Marlin, S.D. *et al.*, *Cell* 51:813 (1987)) (Figure 6A). In contrast, the anti-HLA class I control monoclonal antibody W6/32, which also binds to HUVECs (Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988)), did not inhibit eosinophil adherence (Figure 6A).

35 Adherence of primate lung eosinophils to immune complex coated plastic was not inhibited by RR1/1 (Figure 6B) demonstrating the specificity of the inhibition of adherence to endothelium. These results indicate that ICAM-1 is important for eosinophil adhesion to endothelium and thus may contribute to eosinophil migration into inflamed tissues *in vivo*.

40 In addition to being required for migration into tissue, leukocyte adhesion is a prerequisite for cytotoxic tissue injury. Inhibition of adhesion of effector to target cells has been reported to reduce both lymphocyte and granulocyte mediated killing *in vitro* and allograft rejection *in vivo* (Martz, E. *et al.*, *J. Immunol.* 133:2972 (1984)). Since eosinophils and their products have been implicated in airway epithelium desquamation (Frigas, E. *et al.*, *J. Allergy Clin. Immunol.* 77:527 (1986)) which in turn is strongly linked with airway hyperresponsiveness (Laitinen, L.A. *et al.*, *Am. Rev. Respir. Dis.* 137:62 (1988)) and asthma symptoms (Hargreave, F.E. *et al.*, *J. Allergy Clin. Immunol.* 68:347 (1981); Boulet, L-P *et al.*, *J. Allergy Clin. Immunol.* 71:399 (1983); Chan-Yeung, N. *et al.*, *Am. J. Med.* 72:411 (1982); Frigas, E. *et al.*, *J. Allergy Clin. Immunol.* 77:527 (1986)), the effects of 45 various pro-inflammatory cytokines on ICAM-1 induction on airway epithelial cells *in vitro* was investigated.

48 Using an ELISA assay (Rothlein R. *et al.*, *J. Immunol.* 141:1665 (1988)) and the anti-ICAM-1 monoclonal antibodies RR1/1 and R6.5 (Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988)) it was found that 16 hour stimulation with interleukin-1 beta (IL-1 β), human recombinant tumor necrosis factor alpha (TNF α), and human recombinant interferon gamma (IFN γ) enhanced ICAM-1 expression on a monolayer of cultured monkey bronchus epithelial cells (Table 2).

53 Table 2 shows the effects of pro-inflammatory cytokines on induction of ICAM-1 on bronchial epithelial cells *in vitro*. The rhesus monkey bronchus epithelial cell line 4MBr-5 (obtained from American Type Culture Collection) was cultured to a confluent monolayer and then stimulated for 16 hours with IL-1 β , TNF α or IFN γ . ELISA assays for ICAM-1 [monoclonal antibodies RR1/1 (Rothlein, R. *et al.*, *J. Immunol.* 137:1270 (1986); Marlin, S.D. *et al.*, *Cell* 51:813 (1987)) and R6.5 (Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988))] and LFA-1 alpha [monoclonal antibody R3.1 (Rothlein, R. *et al.*, *J. Immunol.* 141:1665 (1988))] expression were performed as previously described (Rothlein, R. *et al.*, *J. Immunol.* 141:1665 (1988)). The numbers represent the mean of optical density units (relative to normal mouse gamma globulin background) for duplicate cultures and is representative

of four individual experiments.

TABLE 2

Stimulus	Concentration	Optical Density Units		
		RR1/1 (anti-ICAM-1)	R6.5 (anti-ICAM-1)	R3.1 (anti-LFA-1a)
none	---	92	206	-27
IL-1b	0.1 units/ml	130	253	-33
	1 unit /ml	166	253	-32
	10 units/ml	149	322	-37
TNFa	1 unit /ml	100	236	-30
	10 units/ml	131	266	-29
	100 units/ml	159	346	-31
	1000 units/ml	178	416	-36
IFNg	0.1 units/ml	138	276	-16
	1 unit /ml	263	423	-17
	10 units/ml	413	673	-26
	100 units/ml	576	940	-27

The time course of enhanced ICAM-1 expression (Table 3) was found to be similar to that previously reported for HUVECs *in vitro* (Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988)) and human skin keratinocytes *in vivo* (Wantzin, G.L. *et al.*, *J. Am. Acad. Dermatol.* 20:782 (1989)). As expected, the anti-LFA-1 alpha monoclonal antibody R3.1 (Rothlein, R. *et al.*, *J. Immunol.* 141:1665 (1988)) did not bind to unstimulated or stimulated bronchial epithelium (Table 2). These results suggest that ICAM-1 could contribute to leukocyte (e.g., eosinophil) mediated desquamation of airway epithelium *in vivo*.

Table 3 shows the time course for the induction of ICAM-1 on bronchial epithelial cells *in vitro*. The rhesus monkey bronchus epithelial cell line 4MBr-5 (obtained from American Type Culture Collection) was cultured to a confluent monolayer and then stimulated for various time periods with IL-1b (10 ng/ml) or IFNg (10 units/ml). An ELISA assay for ICAM-1 expression [monoclonal antibody R6.5 (Smith, C.W. *et al.*, *J. Clin. Invest.* 82:1746 (1988))] was performed as previously described (Rothlein, R. *et al.*, *J. Immunol.* 141:1665 (1988)). The numbers represent the mean of optical density units (relative to normal mouse gamma globulin background) for triplicate cultures and is representative of two individual experiments.

TABLE 3

Stimulation Time (hours)	Stimulus	
	IL-1b	IFNg
0	215	215
2	240	250
4	296	349
8	364	349
16	417	826
24	369	812
48	472	672

EXAMPLE 3

CONTRIBUTION OF ICAM-1 TO DESQUAMATION IN VIVO

5 To further investigate the ability of ICAM-1 to contribute to eosinophil-mediated desquamation of airway epithelium *in vivo*, immunohistochemical staining was conducted to determine if multiple inhalations of antigen induce ICAM-1 expression on airway epithelium *in vivo*.

10 Tissues were stained using a modification of a previously described protocol (Wantzin, G.L. et al., *J. Am. Acad. Dermatol.* 20:782 (1989)). Briefly, tissue specimens were removed and frozen in liquid nitrogen. After cryo-sectioning, 5-10 micron sections were fixed in acetone for 10 minutes and either stained immediately or stored at -20°C. Staining was performed using the Biotin-Strept Avidin System kit according to manufacturers protocols (BioGenex, CA). Primarily antibody was incubated with tissue as undiluted culture supernatants (RPMI 1640 medium with 10% FBS) for one hour at room temperature. Blocking for non-specific protein binding was accomplished by applying normal goat serum. AEC (3-amino-9-ethylcarbazole) was used as a substrate and the sections were counterstained with Mayer's Hematoxylin.

15 Intense staining for ICAM-1 was found on both the epithelium (basilateral portion only) and on the vascular endothelium of a trachea section taken from an *Ascaris* antigen sensitive monkey twenty minutes after the third of three alternate day *Ascaris* inhalations. Staining for LFA-1 alpha (Anderson, D.C. et al., *J. Inf. Dis.* 152:868 (1985); Anderson, D.C. et al., *Ann. Rev. Med.* 38:175 (1987); Todd, R.F. et al., *Hematol./Oncol. Clinics N. Amer.* 2:13 (1988)) but not on airway epithelium (Table 2) revealed a leukocyte infiltration in the interstitium that 20 appeared to be most marked just below the epithelial basement membrane. In addition leukocytes were notable between epithelial cells primarily at the basilateral portion of the epithelium where ICAM-1 staining was most pronounced. Little or no nonspecific staining was observed using mouse serum. Staining (using ICAM-1, LFA-1 alpha and mouse serum) in a trachea section taken from an *Ascaris*-sensitive monkey twenty minutes after a 25 single inhalation of *Ascaris*, revealed, as expected based on the time required for its expression (see Table 3 and references Wantzin, G.L. et al., *J. Am. Acad. Dermatol.* 20:782 (1989); Smith, C.W. et al., *J. Clin. Invest.* 82:1746 (1988), which references are incorporated herein by reference), little or no ICAM-1 staining was found on the epithelium or vascular endothelium in this section.

30 In addition, although pockets of leukocyte infiltration were found, leukocytes were not accumulated just below the epithelial baseline membrane and were not found between epithelial cells. Thus, these immunohistochemical staining results further suggest an ICAM-1 dependent eosinophil-epithelial cell interaction may contribute to the desquamation of airway epithelium found in asthmatics.

EXAMPLE 4

IN VIVO EFFECT OF ICAM-1 ON ASTHMA

35 Having demonstrated a potential role for ICAM-1 in the pathogenesis of airway hyperresponsiveness and asthma using the *in vitro* and immunohistochemical protocols described above, the *in vivo* effect of anti-ICAM-1 antibodies was investigated. For these studies, an asthma animal model was employed. The model may be used with any mammal, but it is most preferable to employ primates in the model. To induce an asthmatic episode, inhalations of antigen were provided to a monkey on three alternate days. This regimen induced a 40 consistent (usually greater than 8 fold) increase in airway responsiveness to inhaled methacholine in monkeys (Wegner, C.D. et al., *Am. Rev. Respir. Dis.* 139:A324 (1989), which reference is incorporated herein by reference). This increase in airway responsiveness is preceded by an intense eosinophil infiltration and is similar 45 in magnitude to that induced in asthmatics during the pollen season (Boulet, L-P et al., *J. Allergy Clin. Immunol.* 71:399 (1983); Sotomayor, H. et al., *Am. Rev. Respir. Dis.* 130:56 (1984)) or on continued exposure to occupational allergens (Chan-Yeung, M. et al., *Am. J. Med.* 72:411 (1982); Lam, S. et al., *J. Allergy Clin. Immunol.* 72:134 (1983); Lam, S. et al., *J. Allergy Clin. Immunol.* 63:28 (1979)). In lieu of methacholine, histamine or other 50 similar compounds and methacholine equivalents can be employed.

Using this animal model, the effect of the anti-ICAM-1 monoclonal antibody R6.5 on eosinophil infiltration and the induction of airway hyperresponsiveness *in vivo* was investigated.

55 In accordance with the model, airway cell composition and airway responsiveness were determined three days prior to (Day 0) and three days after (Day 10) three alternate day (Day 3, 5 and 7) inhalations of antigen (Wegner, C.D. et al., *Am. Rev. Respir. Dis.* 139:A324 (1989)). Airway cell composition was measured by bronchoalveolar lavage (BAL). Airway responsiveness was measured by determining the concentration (PC₁₀₀) of inhaled methacholine that caused a 100% increase in respiratory system resistance. R6.5 was administered intravenously at 1.76 mg/kg daily on Days 2-9. Studies with R6.5 treatment were compared to bracketing control

studies performed on each animal.

R6.5 (anti-ICAM-1) treatment attenuated the eosinophil infiltration in all five animals studies (Figure 7A). The increase in airway responsiveness (decrease in inhaled methacholine PC₁₀₀) was also inhibited in all five animals, markedly in four (Figure 7B). Surprisingly, in two animals (c and d) airway responsiveness not only did not increase (as it had in control studies) but actually decreased (methacholine PC₁₀₀ increased) with R6.5 treatment, despite the multiple inhalations of antigen, demonstrating a reversal of an elevated basal airway responsiveness in these animals.

In summary, these results demonstrate that ICAM-1 is selectively induced on chronically inflamed tracheal vascular endothelium *in vivo*, that ICAM-1 contributes to eosinophil adhesion to vascular endothelium *in vitro*, and that an anti-ICAM-1 monoclonal antibody attenuates inhaled antigen-induced eosinophil infiltration *in vivo*. Furthermore, ICAM-1 expression is enhanced on cytokine stimulated airway epithelium *in vitro* and selectively induced on chronically inflamed tracheal epithelium *in vivo* indicating that ICAM-1 may contribute to airway epithelium desquamation *in vivo*. An anti-ICAM-1 monoclonal antibody was able to inhibit the increase in airway responsiveness induced by multiple inhalations of antigen in monkeys.

These results indicate that ICAM-1 plays a pivotal role in the pathogenesis of airway hyperresponsiveness and asthma. ICAM-1 similarly contributes to the onset and progression of other diseases characterized by airway inflammation [e.g., chronic bronchitis, emphysema, idiopathic pulmonary fibrosis, etc. (Guenther, C.A. *et al.*, *Am. Rev. Respir. Dis.* 123:79 (1981); Rossi, G.A. *et al.*, *Am. Rev. Respir. Dis.* 129:850 (1984); Hunninghake, G.W. *et al.*, *Am. J. Pathol.* 97:149 (1979); Hunninghake, G.W. *et al.*, *Am. Rev. Respir. Dis.* 123:407 (1981))] or eosinophil infiltration and tissue sensitization / destruction [e.g., rhinitis, nasal polypsis, chronic urticaria and atopic dermatitis (Mygind, N., *Allergy* 34:195 (1979); Mollarkey, M.F. *et al.*, *J. Allergy Clin. Immunol.* 65:122 (1980); Peters, M.S. *et al.*, *J. Invest. Dermatol.* 81:39 (1983); Leberman, K.M. *et al.*, *N. Engl. J. Med.* 313:282 (1985); Spry, C.J.F. *et al.*, *Int. Archs. Allergy appl. Immun.* 77:252 (1985))]. Thus, agents which prevent or attenuate such cellular adhesion may be employed in the treatment of these diseases in the same manner as they may be used to treat asthma.

EXAMPLE 5

EFFECTS OF SINGLE AND MULTIPLE INHALATIONS OF ANTIGEN ON AIRWAY RESPONSIVENESS IN MONKEYS

In animals (Lanes, S. *et al.*, *J. Appl. Physiol.* 61:864-872 (1986), Marsh, W.R. *et al.*, *Amer. Rev. Respir. Dis.* 131:875-879 (1985)), as well as in man (Cartier, A. *et al.*, *J. Allergy Clin. Immunol.* 70:170-177 (1982), Cockcroft, D.W. *et al.*, *Clinical Allergy* 7:503-513 (1977)), a single inhalation of an allergen can cause a mild (2-6 fold) increase in airway responsiveness. Repeated allergen exposures, such as those that occur in man during the pollen season (Boulet, L.-P. *et al.*, *J. Allergy Clin. Immunol.* 71:399-406 (1983), Sotomayor, H. *et al.*, *Amer. Rev. Respir. Dis.* 130:56-58 (1984)) or in an occupational setting (Chan-Yeung, M. *et al.*, *Amer. J. Med.* 72:411-415 (1982), Lam, S. *et al.*, *J. Allergy Clin. Immunol.* 72:134-139 (1983), Lam, S. *et al.*, *J. Allergy Clin. Immunol.* 63:28-34 (1979)), have been reported to cause greater (often \pm 10 fold) increases in airway responsiveness.

Multiple (4 weekly) instillations of antigen coated beads induce a 10 fold increase in airway responsiveness in monkeys (Gundel, R.H. *et al.*, *Amer. Rev. Respir. Dis.* (1989), which reference is incorporated herein by reference).

In order to more develop a useful animal asthma model, the effects of single and multiple antigen inhalations on airway responsiveness to inhaled methacholine in monkeys was investigated. Since airway inflammation (Marsh, W.R. *et al.*, *Amer. Rev. Respir. Dis.* 131:875-879 (1985), Sotomayor, H. *et al.*, *Amer. Rev. Respir. Dis.* 130:56-58 (1984), Lazarus, S., *Amer. J. Med.* 81:2-7 (1986), O'Byrne, P.M., *Chest* 90:575-577 (1986)), especially eosinophil infiltration (Gundel, R.H. *et al.*, *Amer. Rev. Respir. Dis.* (1989), DeMonchy, J.G.R. *et al.*, *Amer. Rev. Respir. Dis.* 131:373-376 (1985), Wardlaw, A.J. *et al.*, *Amer. Rev. Respir. Dis.* 137:62-69 (1988)), has been hypothesized to play a role in the pathogenesis of airway hyperresponsiveness, airway cellular composition was concomitantly investigated.

In order to investigate these effects, seven adult male cynomolgus monkeys (*Macaca fascicularis*, Charles River Primate Imports, Port Washington, NY) weighing 4.6 to 8.2 kg were studied. All animals demonstrated a naturally occurring and reproducible respiratory sensitivity to inhaled *Ascaris suum* extract. Animals were studied anesthetized with ketamine hydrochloride (1 mg/kg, i.m.; Ketaset, Bristol Laboratories) and xylazine (4 mg/kg, i.m.; Rompun, Miles Laboratories, Inc.), intubated with a cuffed endotracheal tube (5.5 mm ID; Mallinckrodt Critical Care, cat #86048) and seated in an upright position in a specially designed support chair. Ketamine (4 mg/kg, i.m.) was used alone to supplement anesthesia as needed.

Airway responsiveness (methacholine PC₁₀₀) followed by airway cell composition (BAL) were determined

one day prior to and twenty hours after a single inhalation of Ascaris extract or three days prior to (Day 0) and three days after (Day 10) three alternate day (Days 3, 5 and 7) inhalations of Ascaris extract. All seven animals were studied in both protocols. A letter designation was assigned to each animal for presentation of results.

In asthmatics, the peak increase in airway responsiveness occurs three to twenty-four hours post allergen challenge (Cartier, A. *et al.*, *J. Allergy Clin. Immunol.* 70:170-177 (1982), Cockcroft, D.W. *et al.*, *Clinical Allergy* 7:503-513 (1977), Durham, S.R. *et al.*, *J. Allergy Clin. Immunol.* 79:398-406 (1987)). Thus, airway responsiveness was measured twenty hours after the single inhalation of antigen.

However, to avoid anesthetizing each animal for a fourth time in a six day interval, airway responsiveness was not measured twenty hours after the multiple inhalation of antigen. Instead, airway responsiveness was measured three days after the multiple inhalations of antigen. It was discovered that airway inflammation (i.e. eosinophil infiltration) still persists three days after antigen inhalation (Wegner, C.D. *et al.*, *Amer. Rev. Respir. Dis.* 135:A221 (1987) which reference is incorporated herein by reference).

Ascaris suum extract (Greer Laboratories, cat #B-33) was employed as antigen in the studies. The extract was diluted in phosphate buffered (5mM, pH 7.4) saline (0.5%) (PBS), compressed air nebulized (Bird Micro-nebulizer, model 8158) and administered by intermittent positive pressure breathing (Bird Mark 7A Respirator) consisting of 30 inhalations to 20 cm H₂O in approximately two minutes. For each animal, the previously determined concentration of Ascaris extract that caused a reproducible 150 to 300% acute increase in respiratory system resistance was used. Methacholine challenges were performed in an identical manner except that they consisted of only 15 breaths in one minute.

Respiratory system impedance (Z_{rs}) was measured by discrete frequency (4-40 Hz in 11 equal logarithmic steps) sinusoidal forced oscillations superimposed on tidal breathing as described by Wegner, C.D. *et al.* (*Respir. Physiol.* 55:47-61 (1983) which reference is incorporated herein by reference). The mean of the real or in-phase component of Z_{rs} over the frequency range was then computed to provide a single value representation of respiratory system resistance (R_{rs}). R_{rs} was measured at 3, 7, 10, 15, 20 and 30 minutes after each antigen challenge and at 1 and 3 minutes after each methacholine challenge.

Airway Responsiveness was assessed by determining the concentration of nebulized and inhaled methacholine that induced a 100% increase in R_{rs} (PC₁₀₀). This was accomplished by administering increasing concentrations of methacholine (diluted with PBS) in half-logarithmic steps (at 7 minute intervals) until a greater than 100% increase in R_{rs} from baseline was obtained. The PC₁₀₀ was then calculated by linear regression analysis of the last two or three points on the logarithm methacholine concentration versus percent increase in R_{rs} plot.

Airway cell composition was assayed by bronchoalveolar lavage (BAL). A pediatric fiberoptic bronchoscope (Olympus Corporation, model BF-3C4) was guided past the carina until it wedged into typically a fifth to seventh generation bronchus. A single 15 ml aliquot of bicarbonate buffered (0.5 mM) normal saline (pH 7.4) was then infused and gently aspirated (return volume range from 7 to 10 ml) through a channel in the bronchoscope. Total leukocytes/ml of BAL was determined using a Coulter counter (Coulter Electronics, model Z_{8i}). Differential cell counts (a total of 200 cells counted) were performed on Wright-Giemsa stained cytocentrifuge (Shandon Cytospin, model 2) preparations. To prevent the effects of a prior BAL on subsequent BAL determinations, the BALs performed prior to and after antigen challenge were done on opposite sides of the lungs.

Analysis of variance was performed using the nonparametric Kruskal-Wallis Test (Chi-square approximation). Correlations were performed using Pearson product-moment and Spearman ranks of the variables. A p value of > 0.05 was considered not significant.

The single inhalation of antigen caused an acute increase in R_{rs} ($307 \pm 62\%$) that peaked 10 - 15 minutes post challenge, an increase in airway leukocytes (267 ± 19 to $694 \pm 142 \times 10^3/\text{ml}$ of BAL, $p > X^2 = 0.018$) and a decrease in methacholine PC₁₀₀ in 3 animals that was moderate (> 8 fold) in two (Figure 8A). The increase in airway leukocytes consisted of an infiltration of eosinophils in all animals (Figure 8B), neutrophils in 3 animals (Figure 8C), macrophages/monocytes in 5 animals (Figure 8D) and lymphocytes in 4 animals (Figure 8E). The magnitude and direction of the change in airway responsiveness (methacholine PC₁₀₀) did not correlate with the intensity of the acute increase in R_{rs} or with the magnitude/existence of the infiltration of any leukocyte subtype (Table 4).

The multiple inhalations of antigen caused acute increases in R_{rs} ($178 \pm 48\%$, $380 \pm 83\%$ and $331 \pm 63\%$, respectively), and increase in airway leukocytes (209 ± 42 to $553 \pm 129 \times 10^3/\text{ml}$ of BAL, $p > X^2 = 0.0088$) and a decrease in methacholine PC₁₀₀ in all 7 animals that was moderate (> 8 fold) in two and severe (> 80 fold) in three (Figure 9A). The increase in airway leukocytes consisted of a marked infiltration of eosinophils (Figure 9B) and slight infiltration of neutrophils (8.5 ± 2.6 to $31.0 \pm 7.5 \times 10^3/\text{ml}$ of BAL, $p > X^2 = 0.010$). There was no significant infiltration of macrophages/monocytes (179 ± 36 to $209 \pm 40 \times 10^3/\text{ml}$ of BAL) or lymphocytes (3.0 ± 1.2 to $3.0 \pm 1.3 \times 10^3/\text{ml}$ of BAL). The magnitude of the increase in airway reactivity (decrease in PC₁₀₀) did not correlate with the intensity of the eosinophil infiltration or with the magnitude/direction of the change in air-

way reactivity (PC₁₀₀) induced by the single inhalation of antigen (Table 5).

TABLE 4

5

10

COMPARISON OF CHANGE IN METHACHOLINE PC₁₀₀ TO THE BRONCHOCONSTRICITION
AND LEUKOCYTE INFILTRATION INDUCED BY A SINGLE INHALATION OF ANTIGEN *

15

Animal	Change in Log. PC ₁₀₀	Increase in Rrs (%)	Leukocyte Infiltration (x10 ³ /ml BAL)			
			Eosin.	Neut.	Mac./Mono.	Lympho.
a	-1.02	494	23	297	105	3.3
b	-0.95	278	266	-33	421	20.5
c	-0.75	181	668	87	104	2.7
d	0.16	244	242	6	253	18.6
e	0.21	525	38	-6	-73	-1.6
f	0.24	66	124	-2	-78	-3.0
g	0.36	360	221	178	264	-0.4
Mean	-0.25	307	226	76	142	5.8
S.E.	±0.24	±63	±82	±46	±69	±3.7

Definition of abbreviations: Log. = logarithm base 10; PC₁₀₀ = provocative concentration of methacholine required to cause a 100% increase in Rrs; Rrs = respiratory system resistance; BAL = bronchoalveolar lavage; Eosin. = eosinophils; Neut. = neutrophils; Mac./Mono. = macrophages/monocytes; Lympho. = lymphocytes.

* No significant correlations were found.

40

45

50

55

TABLE 5

5

COMPARISON OF CHANGE IN METHACHOLINE PC₁₀₀ TO EOSINOPHIL INFILTRATION
INDUCED BY MULTIPLE INHALATIONS OF ANTIGEN TO THE CHANGE IN METHACHOLINE
PC₁₀₀ INDUCED BY A SINGLE INHALATION OF ANTIGEN *

10

15

20

25

30

	Animal	Multiple Inhalations of Antigen		Single Inhalation of Ag. Change in Log. PC ₁₀₀
		Change in Log. PC ₁₀₀	Eosin. Infiltration ($\times 10^3$ /ml BAL)	
d	d	-2.66	144	0.16
	b	-2.13	352	-0.95
	e	-1.95	409	0.21
	a	-1.07	135	-1.02
	g	-0.97	174	0.36
	c	-0.43	206	-0.75
	f	-0.27	109	0.24
Mean		-1.36	218	-0.25
S.E.		±0.34	±44	±0.24

Definition of abbreviations: Log. = logarithm base 10; PC₁₀₀ = provocative concentration of methacholine required to cause a 100% increase in respiratory system resistance; BAL = bronchoalveolar lavage; Eosin. = eosinophils; Ag. = antigen.

* No significant correlations were found.

These experiments demonstrate that non-specific airway hyper-responsiveness, as assessed clinically by responsiveness of the airways to inhaled histamine, methacholine, exercise or cold air, is a characteristic feature of asthma (Boushey, H.A. *et al.*, *Am. Rev. Respir. Dis.* **121**:389-413 (1980), Hargrave, F.E. *et al.*, *J. Allergy Clin. Immunol.* **68**:347-355 (1981)). While the mechanisms underlying the pathogenesis of airway hyperresponsiveness are not known, results from many studies suggest that leukocyte infiltration (Gundel, R.H. *et al.*, *Amer. Rev. Respir. Dis.* (1989), Marsh, W.R. *et al.*, *Amer. Rev. Respir. Dis.* **131**:875-879 (1985), Lazarus, S., *Amer. J. Med.* **81**:2-7 (1986), O'Byrne, P.M., *Chest* **90**:575-577 (1986), DeMonchy J.G.R. *et al.*, *Amer. Rev. Respir. Dis.* **131**:373-376 (1985), Wardlaw, A.J. *et al.*, *Amer. Rev. Respir. Dis.* **137**:62-69 (1988), Metzger, W.J. *et al.*, *Chest* **89**:477-483 (1986)) and/or mediators released by resident or infiltrating cells (Lanes, S. *et al.*, *J. Appl. Physiol.* **61**:864-872 (1986), Lazarus, S., *Amer. J. Med.* **81**:2-7 (1986) O'Byrne, P.M., *Chest* **90**:575-577 (1986), Wardlaw, A.J. *et al.*, *Amer. Rev. Respir. Dis.* **137**:62-69 (1988), Aizawa, H. *et al.*, *J. Appl. Physiol.* **59**:1918-1923 (1985), O'Byrne, P.M. *et al.*, *Prostaglandins* **4**:537-543 (1984), Schulman, E.S., *Chest* **90**:578-586 (1986)) are involved.

Antigen inhalation is known to induce an acute neutrophil and more chronic eosinophil airway infiltration (Wegner, C.D. *et al.*, *Amer. Rev. Respir. Dis.* **135**:A221 (1987)). A chronic idiopathic airway eosinophilia has been found to be associated with severe (> 80 fold) airway hyperresponsiveness (Wegner, C.D. *et al.*, *Amer. Rev. Respir. Dis.* **135**:A222 (1987)). Multiple (four weekly) intratracheal instillations of antigen-coated beads have been found to induce marked increases in airway eosinophils and responsiveness (> 8 fold) (Gundel, R.H. *et al.*, *Amer. Rev. Respir. Dis.* (1989)). The experiments presented above provide a comparison of the effects of a single versus multiple (three alternate day) inhalations of *Ascaris suum* extract on airways responsiveness and leukocyte composition in "allergic" monkeys.

The single inhalation of antigen resulted in an acute bronchoconstriction and, as measured 20 hours later, an infiltration of leukocytes (primarily and most consistently eosinophils) as well as an increase in airway responsiveness (decrease in inhaled methacholine PC₁₀₀) in 3 of 7 animals that was moderate (>8 fold) in two. These effects, as well as the frequency which they occurred, are similar to those reported for man. That is, in

allergic asthmatics a single inhalation of antigen induces a predominately eosinophilic leukocyte infiltration (DeMonchy, J.G.R. *et al.*, *Amer. Rev. Respir. Dis.* **131**:373-376 (1985)) and an increase (usually mild, <8 fold) in airway responsiveness in some individuals (Cartier, A. *et al.*, *J. Allergy Clin. Immunol.* **70**:170-177 (1982), Cockcroft, D.W. *et al.*, *Clinical Allergy* **7**:503-513 (1977)).

5 In contrast, the multiple inhalations of antigen induced, as assayed three days after the last challenge, an increase in airway responsiveness in all seven monkeys that was moderate (>8 fold) in two and sever (>80 fold) in three. These effects are also consistent with those reported for man. During the pollen season, the airway responsiveness of allergic asthmatics has been reported to increase in all individuals, although to varying degrees (Boulet, L.-P. *et al.*, *J. Allergy. Clin. Immunol.* **71**:399-406 (1983), Sotomayor, H. *et al.*, *Amer. Rev. Respir. Dis.* **130**:56-58 (1984)). Similarly, repeated exposure to occupational allergens has been shown to increase airway responsiveness in sensitive individuals (Chan-Yeung M., *et al.*, *Amer. J. Med.* **72**:411-415 (1982), Lam, S. *et al.*, *J. Allergy Clin. Immunol.* **72**:134-139 (1983), Lam, S. *et al.*, *J. Allergy Clin. Immunol.* **63**:28-34 (1979)).

10 In both the single and multiple inhalation of antigen protocols, the magnitude/direction of change in airway responsiveness did not correlate with the intensity of the leukocyte infiltration. This finding emphasizes the leukocyte infiltration alone is not enough to infer their direct involvement in the pathogenesis of airway hyper-reactivity. Rather, the amount and type (stimulator or inhibitor) of mediators released by the infiltrating and resident cells as well as the interactions occurring between the infiltrating and resident cells (e.g., eosinophils and airway epithelium) are important factors.

15 In summary, as has been previously reported for asthmatics, multiple (but not single) inhalations of antigen induce a consistent (usually > 8 fold) increase in airway responsiveness in monkeys. These findings indicate that this animal model can be used to screen for and identify agents capable of providing a therapy for asthma.

20 While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, 25 or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

30

Claims

1. The use of an agent selected from
 - (a) an antibody capable of binding to ICAM-1;
 - (b) a fragment of said antibody (a), said fragment being capable of binding to ICAM-1;
 - (c) ICAM-1, being substantially free of natural contaminants;
 - (d) a functional derivative of ICAM-1;
 - (e) an antibody capable of binding to a member of the CD18 family of glycoproteins;
 - (f) a fragment of said antibody (e), said fragment being capable of binding to a member of the CD18 family of glycoproteins;
 - (g) a member of the CD18 family of glycoproteins, being substantially free of natural contaminants; and
 - (h) a functional derivative of a member of the CD18 family of glycoproteins.
2. The use of claim 1 wherein said agent is said antibody (a) capable of binding to ICAM-1, or a fragment of said antibody (a), said fragment being capable of binding to ICAM-1.
3. The use of claim 2 wherein said antibody (a) is a monoclonal antibody.
4. The use of claim 3 wherein said monoclonal antibody is the monoclonal antibody R6.5.
5. The use of claim 1 wherein said agent is ICAM-1, being substantially free of natural contaminants.
6. The use of claim 1 wherein said agent is a functional derivative of ICAM-1.
7. The use of claim 6 in which the functional derivative contains ICAM-1 domain 1.
8. The use of claim 6 in which the functional derivative contains ICAM-1 domain 2.
9. The use of claim 6 in which the functional derivative is a soluble derivative of ICAM-1.
10. The use of claim 1 wherein said agent is said antibody (e) capable of binding to a member of the CD18 family of glycoproteins; or a fragment of said antibody (e), said fragment being capable of binding to a member of the CD18 family of glycoproteins.
11. The use of claim 10 wherein said antibody (e) is able to bind to an alpha subunit of said member of the CD18 family of glycoproteins.
12. The use of claim 10 wherein said antibody (e) is able to bind to a beta subunit of said member of the

CD18 family of glycoproteins.

13. The use of claim 1 wherein said agent is a member of the CD18 family of glycoproteins, being substantially free of natural contaminants.

14. The use of claim 13 wherein said member of the CD18 family of glycoproteins contains an alpha subunit of a member of the CD18 family of glycoproteins.

5 15. The use of claim 13 wherein said member of the CD18 family of glycoproteins contains a beta subunit of a member of the CD18 family of glycoproteins.

16. The use of claim 13 wherein said member of the CD18 family of glycoproteins is a heterodimer containing both an alpha and a beta subunit of a member of the CD18 family of glycoproteins.

10 17. The use of claim 13 wherein said agent is a functional derivative of a member of the CD18 family of glycoproteins.

18. A method of identifying an agent capable of therapeutic potential in the treatment of asthma which comprises administering said agent to a non-human mammal having received multiple inhalations of an antigen, and measuring any increase in airway responsiveness of said mammal.

15 19. The method of claim 18 wherein any increase in airway responsiveness in said mammal is measured with methacholine or histamine.

Patentansprüche

20 1. Verwendung eines Wirkstoffes ausgewählt aus

- (a) einem Antikörper, der fähig ist, ICAM-1 zu binden;
- (b) einem Fragment des besagten Antikörpers (a), wobei besagtes Fragment fähig ist, ICAM-1 zu binden;
- (c) ICAM-1, welches im wesentlichen frei von natürlichen Verunreinigungen ist;
- (d) einem funktionellen Derivat von ICAM-1;

25 (e) einem Antikörper, der fähig ist, ein Mitglied der CD18-Familie der Glykoproteine zu binden;

- (f) einem Fragment des besagten Antikörpers (e), welches zur Bindung an ein Mitglied der CD18-Familie der Glykoproteine fähig ist;
- (g) einem Mitglied der CD18-Familie der Glykoproteine, welches im wesentlichen frei von natürlichen Verunreinigungen ist; und

30 (h) einem funktionellen Derivat eines Mitglieds der CD18-Familie der Glykoproteine in der Herstellung eines pharmazeutischen Präparates zur Behandlung von Asthma.

2. Verwendung nach Anspruch 1, worin der besagte Wirkstoff der besagte Antikörper (a) ist, der zur Bindung an ICAM-1 befähigt ist, oder ein Fragment des besagten Antikörpers (a) ist, das zur Bindung an ICAM-1 befähigt ist.

35 3. Verwendung nach Anspruch 2, worin der besagte Antikörper (a) ein monoklonaler Antikörper ist.

4. Verwendung nach Anspruch 3, worin der besagte monoklonale Antikörper der monoklonale Antikörper R6.5 ist.

5. Verwendung nach Anspruch 1, worin der besagte Wirkstoff ICAM-1 ist, welches im wesentlichen frei von natürlichen Verunreinigungen ist.

40 6. Verwendung nach Anspruch 1, worin der besagte Wirkstoff ein funktionelles Derivat von ICAM-1 ist.

7. Verwendung nach Anspruch 6, worin das funktionelle Derivat die ICAM-1 Domäne 1 enthält.

8. Verwendung nach Anspruch 6, worin das funktionelle Derivat die ICAM-1 Domäne 2 enthält.

9. Verwendung nach Anspruch 6, worin das funktionelle Derivat ein lösliches Derivat von ICAM-1 ist.

45 10. Verwendung nach Anspruch 1, worin der besagte Wirkstoff der besagte Antikörper (e) ist, der zur Bindung an ein Mitglied der CD18-Familie der Glykoproteine befähigt ist; oder ein Fragment des besagten Antikörpers (e), wobei dieses Fragment zur Bindung an ein Mitglied der CD18-Familie der Glykoproteine befähigt ist.

11. Verwendung nach Anspruch 10, worin der besagte Antikörper (e) zur Bindung an eine alpha-Untereinheit des besagten Mitglieds der CD18-Familie der Glykoproteine befähigt ist.

50 12. Verwendung nach Anspruch 10, worin der besagte Antikörper (e) zur Bindung an eine beta-Untereinheit des besagten Mitglieds der CD18-Familie der Glykoproteine befähigt ist.

13. Verwendung nach Anspruch 1, worin der besagte Wirkstoff ein Mitglied der CD18-Familie der Glykoproteine ist, das im wesentlichen frei von natürlichen Verunreinigungen ist.

55 14. Verwendung nach Anspruch 13, worin das besagte Mitglied der CD18-Familie der Glykoproteine eine alpha-Untereinheit eines Mitglieds der CD18-Familie der Glykoproteine enthält.

15. Verwendung nach Anspruch 13, worin das besagte Mitglied der CD18-Familie der Glykoproteine eine beta-Untereinheit eines Mitglieds der CD18-Familie der Glykoproteine enthält.

16. Verwendung nach Anspruch 13, worin das besagte Mitglied der CD18-Familie der Glykoproteine ein Heterodimer mit sowohl einer alpha- wie auch einer beta-Untereinheit eines Mitglieds der CD18-Familie der Glykoproteine ist.

17. Verwendung nach Anspruch 13, worin der besagte Wirkstoff ein funktionelles Derivat eines Mitglieds der CD18-Familie der Glykoproteine ist.

5 18. Verfahren zur Identifizierung eines Wirkstoffes, der fähig ist, ein therapeutisches Potential bei der Behandlung von Asthma zu entfalten, wobei der besagte Wirkstoff einem nicht-menschlichen Säugetier, welches mehrfache Antigeninhalationen erhalten hat, verabreicht und die Zunahme der Luftwegreagibilität des besagten Tieres gemessen wird.

10 19. Verfahren nach Anspruch 18, worin eine jegliche Erhöhung der Luftwegreagibilität bei besagtem Tier mit Methacholin oder Histamin gemessen wird.

Revendications

15 1. Utilisation d'un agent choisi parmi

- (a) un anticorps capable de se lier à ICAM-1;
- (b) un fragment dudit anticorps (a), ledit fragment étant capable de se lier à ICAM-1;
- (c) ICAM-1, sensiblement exempte de contaminants naturels;
- (d) un dérivé fonctionnel de ICAM-1;

20 (e) un anticorps capable de se lier à un élément de la famille CD18 des glycoprotéines;

- (f) un fragment dudit anticorps (e), ledit fragment étant capable de se lier à un élément de la famille CD18 des glycoprotéines;
- (g) un élément de la famille CD18 des glycoprotéines, sensiblement exempt de contaminants naturels; et
- (h) un dérivé fonctionnel d'un élément de la famille CD18 des glycoprotéines,

25 dans la fabrication d'une préparation pharmaceutique pour traiter l'asthme.

2. Utilisation selon la revendication 1, dans laquelle ledit agent est ledit anticorps (a) capable de se lier à ICAM-1, ou un fragment dudit anticorps (a), ledit fragment étant capable de se lier à ICAM-1.

3. Utilisation selon la revendication 2, dans laquelle ledit anticorps (a) est un anticorps monoclonal.

30 4. Utilisation selon la revendication 3, dans laquelle ledit anticorps monoclonal est l'anticorps monoclonal R6.5.

5. Utilisation selon la revendication 1, dans laquelle ledit agent est ICAM-1, sensiblement exempte de contaminants naturels.

35 6. Utilisation selon la revendication 1, dans laquelle ledit agent est un dérivé fonctionnel de ICAM-1.

7. Utilisation selon la revendication 6, dans laquelle le dérivé fonctionnel contient le domaine 1 de ICAM-1.

8. Utilisation selon la revendication 6, dans laquelle le dérivé fonctionnel contient le domaine 2 de ICAM-1.

9. Utilisation selon la revendication 6, dans laquelle le dérivé fonctionnel est un dérivé soluble de ICAM-1.

40 10. Utilisation selon la revendication 1, dans laquelle ledit agent est ledit anticorps (e) capable de se lier à un élément de la famille CD18 des glycoprotéines, ou un fragment dudit anticorps (e), ledit fragment étant capable de se lier à un élément de la famille CD18 des glycoprotéines.

11. Utilisation selon la revendication 10, dans laquelle ledit anticorps (e) est capable de se lier à une sous-unité alpha dudit élément de la famille CD18 des glycoprotéines.

45 12. Utilisation selon la revendication 10, dans laquelle ledit anticorps (e) est capable de se lier à une sous-unité bêta dudit élément de la famille CD18 des glycoprotéines.

13. Utilisation selon la revendication 1, dans laquelle ledit agent est un élément de la famille CD18 des glycoprotéines, sensiblement exempt de contaminants naturels.

50 14. Utilisation selon la revendication 13, dans laquelle ledit élément de la famille CD18 des glycoprotéines contient une sous-unité alpha d'un élément de la famille CD18 des glycoprotéines.

15. Utilisation selon la revendication 13, dans laquelle ledit élément de la famille CD18 des glycoprotéines contient une sous-unité bêta d'un élément de la famille CD18 des glycoprotéines.

55 16. Utilisation selon la revendication 13, dans laquelle ledit élément de la famille CD18 des glycoprotéines est un hétérodimère contenant une sous-unité alpha et une sous-unité bêta d'un élément de la famille CD18 des glycoprotéines.

17. Utilisation selon la revendication 13, dans laquelle ledit agent est un dérivé fonctionnel d'un élément de la famille CD18 des glycoprotéines.

18. Procédé d'identification d'un agent capable de pouvoir thérapeutique dans le traitement de l'asthme, qui comprend l'administration dudit agent à un mammifère non humain qui a reçu des inhalations multiples d'un antigène, et la mesure de toute augmentation de la sensibilité des voies aériennes dudit mammifère.

19. Procédé selon la revendication 18, dans lequel toute augmentation de la sensibilité des voies aériennes chez ledit mammifère est mesurée avec la méthacholine ou l'histamine.

5

10

15

20

25

30

35

40

45

50

55

Figure 1

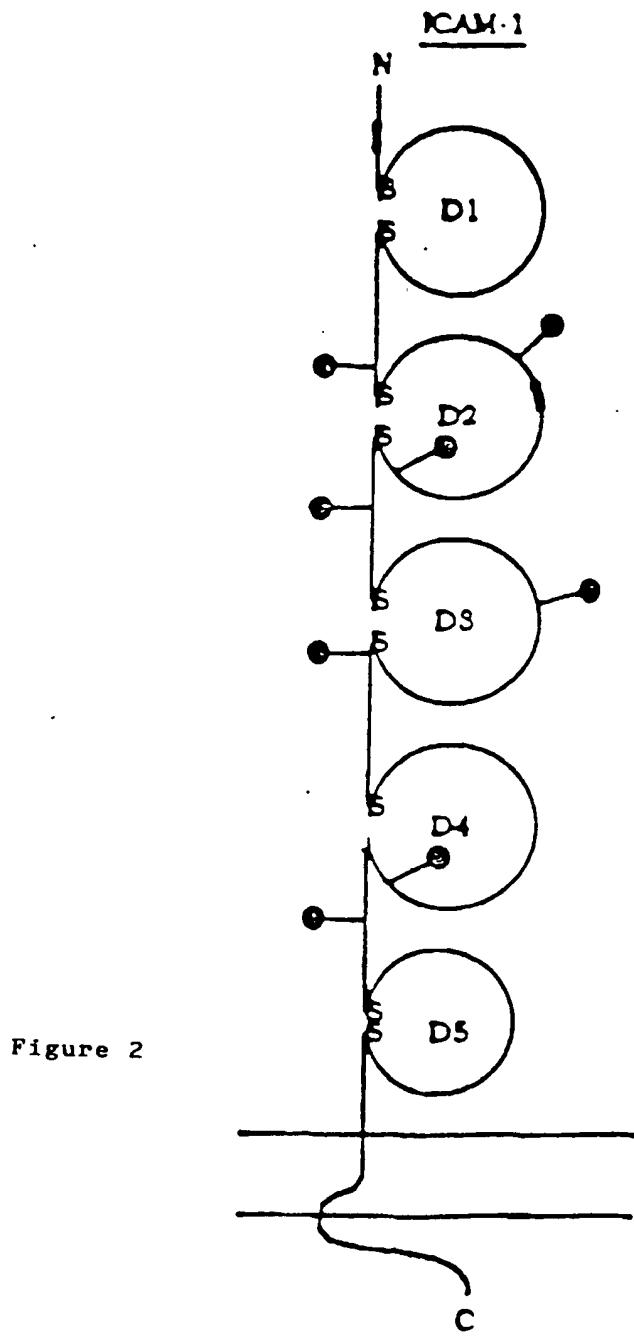


Figure 3

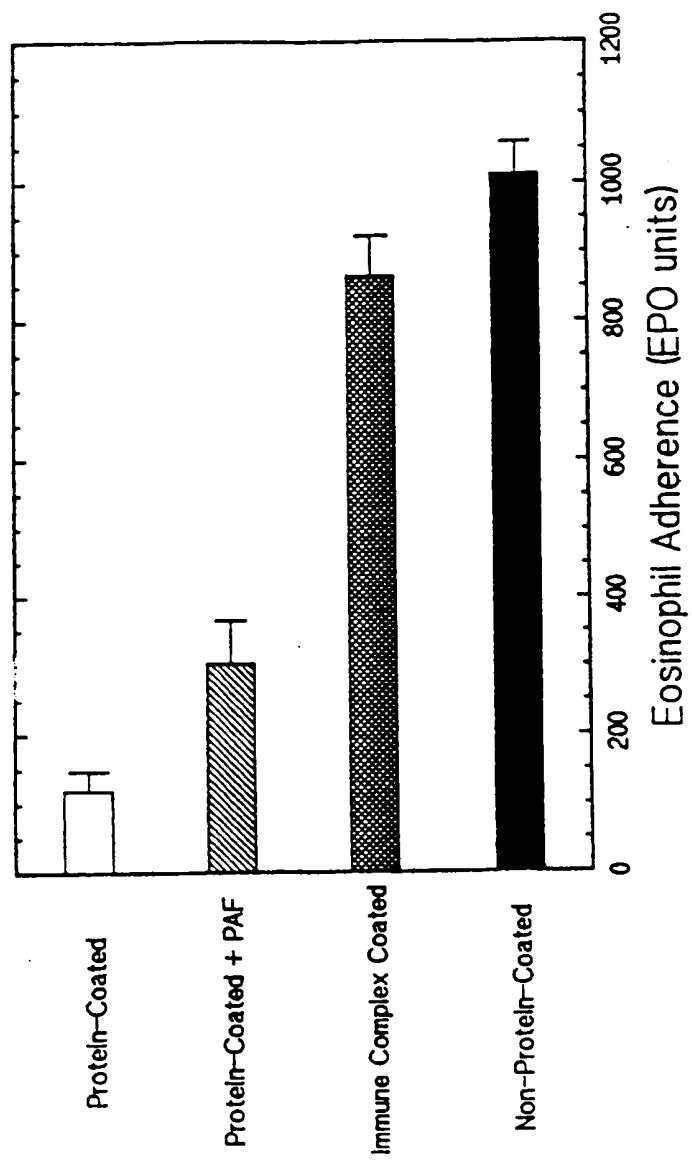


Figure 4

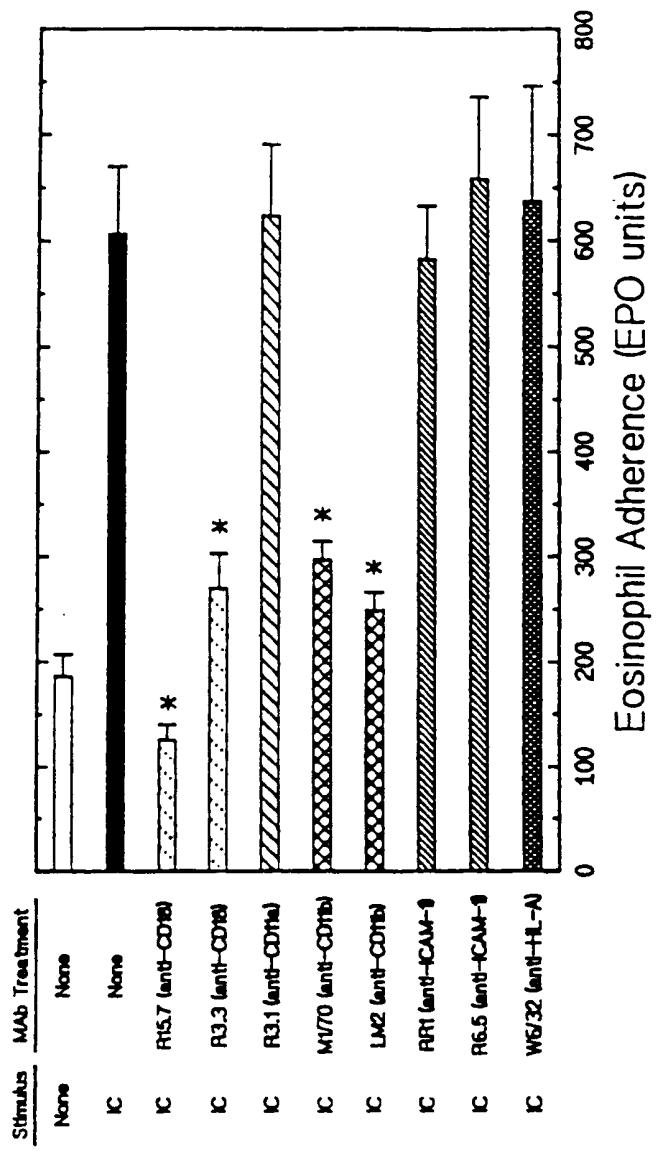


Figure 5

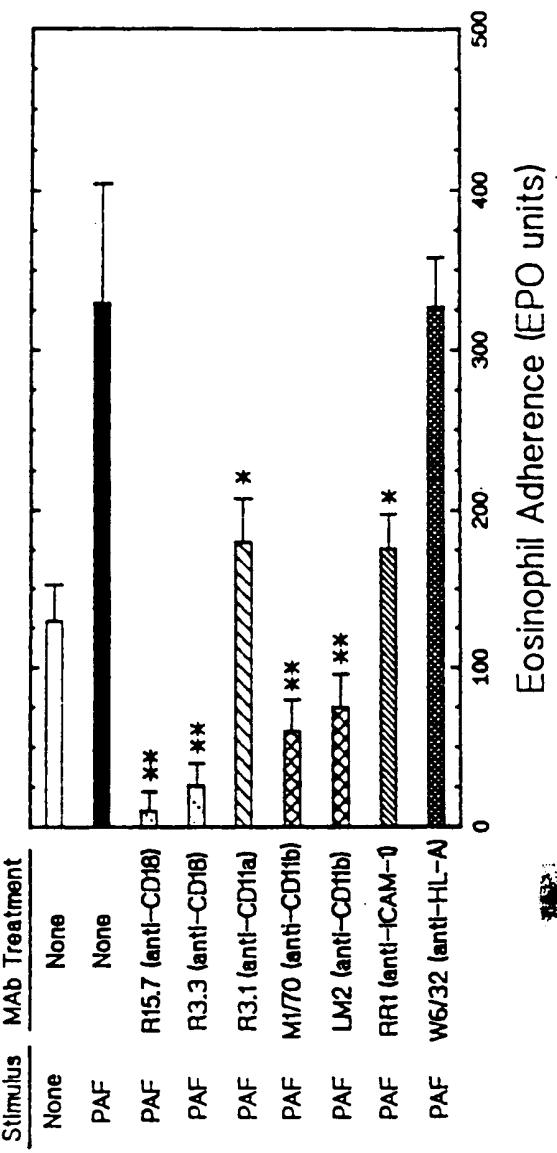
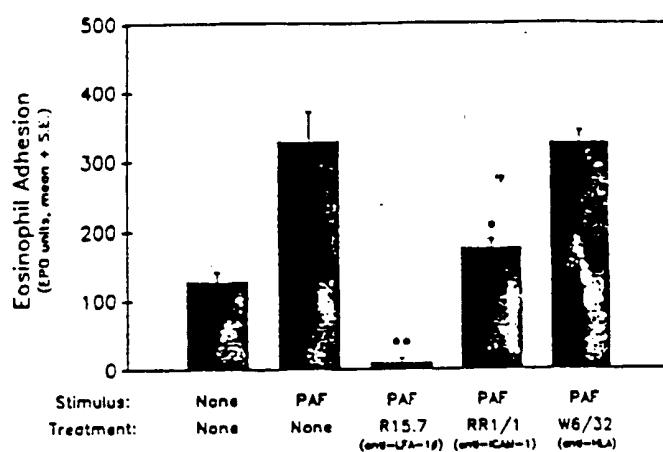
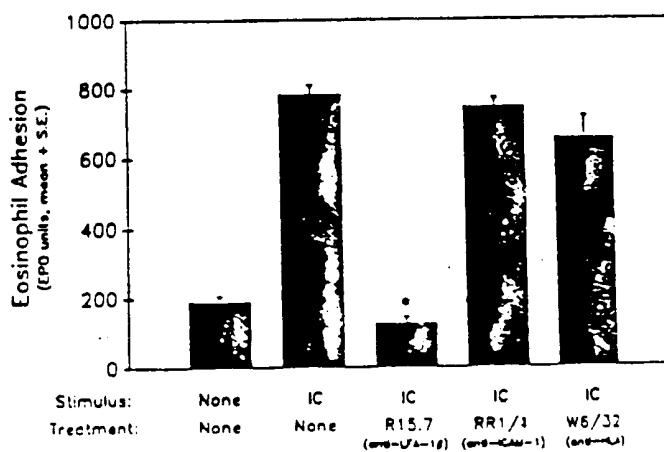


Figure 6

A.



B.



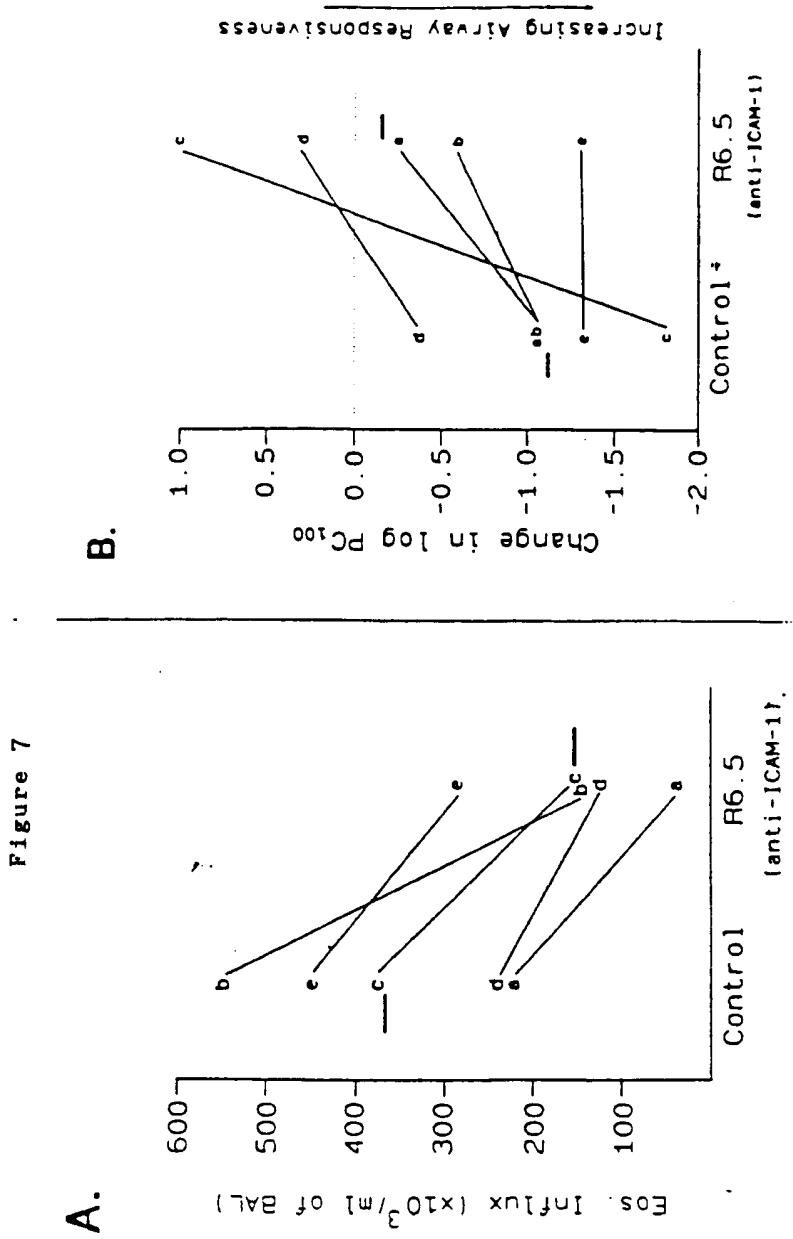


Figure 8

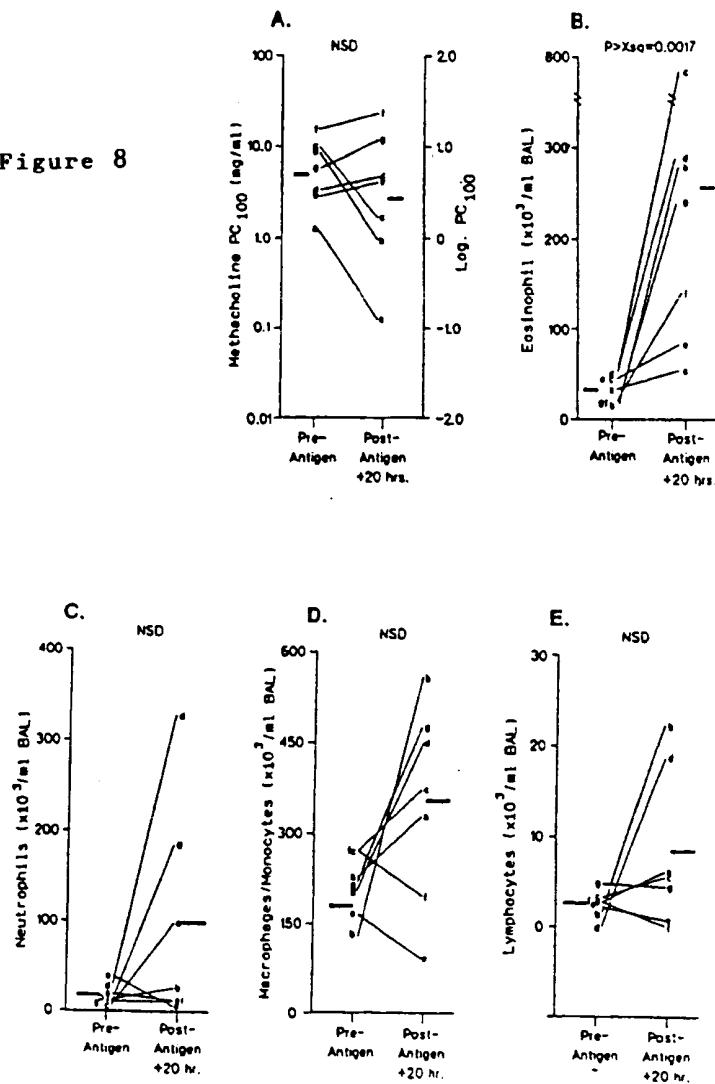


Figure 9

